

89

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number  
**WO 02/29029 A2**

(51) International Patent Classification<sup>7</sup>: **C12N 15/00**

(21) International Application Number: **PCT/EP01/09723**

(22) International Filing Date: **23 August 2001 (23.08.2001)**

(25) Filing Language: **English**

(26) Publication Language: **English**

(30) Priority Data:  
100 48 603.7 30 September 2000 (30.09.2000) DE  
101 09 691.7 28 February 2001 (28.02.2001) DE  
101 36 986.7 28 July 2001 (28.07.2001) DE

(71) Applicant: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1,  
40474 Düsseldorf (DE).

(72) Inventors: **FARWICK, Mike**; Gustav-Adolf-Strasse  
11, 33615 Bielefeld (DE). **HUTHMACHER, Klaus**;  
Lärchenweg 18, 63584 Gelnhausen (DE). **SCHISCHKA,**  
**Natalie**; Stuttgarter Strasse 1, 33659 Bielefeld (DE).  
**BATHE, Brigitte**; Twieten 1, 33154 Salzkotten (DE).  
**PFEFFERLE, Walter**; Jahnstrasse 33, 33790 Halle  
(Westf.) (DE). **BINDER, Michael**; Kalberkamp 28,  
33803 Steinhagen (Westf.) (DE). **GREISSINGER, Di-**  
**eter**; Augasse 1f, 61194 Niddatal (DE). **THIERBACH,**  
**Georg**; Gunststrasse 21, 33613 Bielefeld (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,  
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA,  
ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,  
TG).

**Published:**

— *without international search report and to be republished  
upon receipt of that report*

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*



**WO 02/29029 A2**

(54) Title: **NUCLEOTIDE SEQUENCES WHICH CODE FOR THE CYSD, CYSN, CYSK, CYSE AND CYSH GENES**

(57) Abstract: The invention provides nucleotide sequences from coryneform bacteria which code for the *cysD*, *cysN*, *cysK*, *cysE* and *cysH* genes and a process for the fermentative preparation of amino acids using bacteria in which the genes mentioned are enhanced, a process for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the *cysD* gene, the *cysN* gene, the *cysK* gene, the *cysE* gene and/or the *cysH* gene is present in enhanced form, and the use of polynucleotides which contain the sequences according to the invention as hybridization probes and a process for the preparation of an L-methionine-containing animal feedstuffs additive from fermentation broths.

**Nucleotide Sequences which Code for the cysD, cysN, cysK,  
cysE and cysH Genes**

Field of the Invention

The invention provides nucleotide sequences from coryneform  
5 bacteria which code for the cysD, cysN, cysK, cysE and cysH  
genes and a process for the fermentative preparation of  
amino acids using bacteria in which the endogene genes  
mentioned are enhanced.

Prior Art

10 L-Amino acids, in particular L-lysine, L-cysteine and L-  
methionine, are used in human medicine and in the  
pharmaceuticals industry, in the foodstuffs industry and  
very particularly in animal nutrition.

It is known that amino acids are prepared by fermentation  
15 from strains of coryneform bacteria, in particular  
Corynebacterium glutamicum. Because of their great  
importance, work is constantly being undertaken to improve  
the preparation processes. Improvements to the process can  
relate to fermentation measures, such as, for example,  
20 stirring and supply of oxygen, or the composition of the  
nutrient media, such as, for example, the sugar  
concentration during the fermentation, or the working up to  
the product form by, for example, ion exchange  
chromatography, or the intrinsic output properties of the  
25 microorganism itself.

Methods of mutagenesis, selection and mutant selection are  
used to improve the output properties of these  
microorganisms. Strains which are resistant to  
antimetabolites or are auxotrophic for metabolites of  
30 regulatory importance and produce amino acids are obtained  
in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of Corynebacterium strains which produce L-amino acid, by amplifying individual amino acid biosynthesis genes and  
5 investigating the effect on the amino acid production.

#### Object of the Invention

The inventors had the object of providing new measures for improved fermentative preparation of amino acids.

#### Summary of the Invention

10 Where L-amino acids or amino acids are mentioned in the following, this means one or more amino acids, including their salts, chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-  
15 isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-arginine. L-Lysine and the sulfur-containing L-amino acids L-cysteine and L-methionine are particularly preferred.

When L-lysine or lysine are mentioned in the following, not  
20 only the bases but also the salts, such as e.g. lysine monohydrochloride or lysine sulfate, are meant by this.

When L-cysteine or cysteine are mentioned in the following, the salts, such as e.g. cysteine hydrochloride or cysteine S-sulfate are also meant by this.

25 When L-methionine or methionine are mentioned in the following, the salts, such as e.g. methionine hydrochloride or methionine sulfate are also meant by this.

The invention provides isolated polynucleotides from coryneform bacteria comprising one or more of the  
30 polynucleotide sequences which code for the cysD gene, the

cysN gene, the cysK gene, the cysE gene or the cysH gene, chosen from the group consisting of

- 5 a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
- 10 b) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 3,
- c) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 5,
- 15 d) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 6,
- 20 e) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 8,
- 25 f) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,
- 30 g) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 3,

- h) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 5,
- 5 i) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 6,
- 10 j) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 8,
- k) polynucleotide which is complementary to the polynucleotides of a), b), c), d), e), f), g), h), i) or j), and
- 15 l) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b), c), d), e), f), g), h), i), j) or k),

the polypeptides preferably having the corresponding activities, namely of sulfate adenylyl transferase, 20 cysteine synthase A, serine acetyl transferase or 3'-phosphoadenylyl sulfate reductase.

The invention also provides the above-mentioned polynucleotides, these preferably being DNAs which are 25 capable of replication, comprising:

- (i) one or more nucleotide sequences shown in SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7, or
- (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration 30 of the genetic code, or

- (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
- (iv) sense mutations of neutral function in (i).

5 The invention also provides

polynucleotides, in particular DNAs, which are capable of replication and comprise one or more nucleotide sequences as shown in SEQ ID No. 1, SEQ ID No.4, or SEQ ID No. 7;

10 polynucleotides which code for one or more polypeptides which comprises the corresponding amino acid sequences, as shown in SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 6, or SEQ ID No. 8;

15 a vector containing one or more of the polynucleotides according to the invention, in particular shuttle vectors or plasmid vectors, and

coryneform bacteria which contain the vector or in which one or more of the endogene genes chosen from the group consisting of the cysD gene, cysN gene, cysK gene, cysE gene and cysH gene is/are enhanced.

20

The invention also provides a process for the fermentative preparation of amino acids using bacteria in which one or more endogene genes chosen from the group consisting of

- 25 • the cysD gene which codes for the subunit II of sulfate adenylyltransferase,
- the cysN gene which codes for the subunit I of sulfate adenylyl transferase,
- the cysK gene which codes for cysteine synthase A,
- the cysE gene which codes for serine acetyl transferase,



- the cysH gene which codes for 3'-phosphoadenylyl sulfate reductase

is enhanced.

All five endogene genes (cysD gene, cysN gene, cysK gene, cysE gene and cysH gene) participate in the biosynthesis of the sulfur-containing L-amino acids L-cysteine and L-methionine. The carbon matrix of these amino acids is predominantly derived from the same metabolic intermediates as that of the amino acids of the aspartate family, to which L-lysine belongs. Over-expression of one or more of the genes mentioned leads to pool shifts in the participating biosynthesis pathways, which has a positive effect on the formation of L-lysine, L-methionine and L-cysteine.

The invention also provides polynucleotides which substantially comprise a polynucleotide sequence, which are obtainable by screening by means of hybridization of a corresponding gene library of a coryneform bacterium, which comprises the complete gene or parts thereof, with a probe which comprises the sequence of the polynucleotides according to the invention according to SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7 or a fragment thereof, and isolation of the polynucleotide sequence mentioned.

#### Detailed Description of the Invention

Polynucleotides which comprise the sequences according to the invention are suitable as hybridization probes for RNA, cDNA and DNA, in order to isolate, in the full length, nucleic acids or polynucleotides or genes which code for sulfate adenylyl transferase, cysteine synthase A, serine acetyl transferase and/or 3'-phosphoadenylyl sulfate reductase, or to isolate those nucleic acids or polynucleotides or genes which have a high similarity of

sequence with that of the cysD gene, the cysN gene, the cysK gene, the cysE gene and/or the cysH gene.

Polynucleotides which comprise the sequences according to the invention are furthermore suitable as primers with the aid of which DNA of genes which code for sulfate adenylyl transferase, cysteine synthase A, serine acetyl transferase and/or 3'-phosphoadenylyl sulfate reductase can be prepared by the polymerase chain reaction (PCR).

In one aspect of this invention, the cysD gene according to the invention codes for the subunit II of sulfate adenylyl transferase, the cysN gene according to the invention codes for the subunit I of sulfate adenylyl transferase, the cysK gene according to the invention codes for cysteine synthase A, the cysE gene according to the invention codes for serine acetyl transferase and the cysH gene according to the invention codes for 3'-phosphoadenylyl sulfate reductase.

In another aspect of this invention, it is possible that these genes according to the invention occur in pairs or in combination with several genes, in which case they then code for the combined activities. That is to say, if, for example, the a) cysE gene and cysK gene, or b) cysK gene and cysH gene, or c) cysN gene and cysD gene and cysE gene and cysK gene are enhanced at the same time, these code for a) serine acetyl transferase and cysteine synthase A, b) cysteine synthase A and 3'-phosphoadenylyl sulfate reductase, and c) sulfate adenylyl transferase and serine acetyltransferase and cysteine synthase A.

Such oligonucleotides which serve as probes or primers comprise at least 30, preferably at least 20, very particularly preferably at least 15 successive nucleotides. Oligonucleotides which have a length of at least 40 or 50 nucleotides are also suitable. Oligonucleotides with a



length of at least 100, 150, 200, 250 or 300 nucleotides are optionally also suitable.

"Isolated" means separated out of its natural environment.

5 "Polynucleotide" in general relates to polyribonucleotides and polydeoxyribonucleotides, it being possible for these to be non-modified RNA or DNA or modified RNA or DNA.

10 The polynucleotides according to the invention include a polynucleotide according to SEQ ID No. 1, SEQ ID No. 4, or SEQ ID No. 7 or a fragment prepared therefrom and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the polynucleotide according to SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7 or a fragment prepared therefrom.

15 "Polypeptides" are understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

20 The polypeptides according to the invention include the polypeptides according to SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 6 and SEQ ID No. 8, in particular those with the biological activity of sulfate adenylyl transferase, cysteine synthase A, serine acetyl transferase and/or 3'-phosphoadenylyl sulfate reductase, and also those which are at least 70%, preferably at least 80% and in particular at least 90% to 95% identical to the  
25 polypeptides according to SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 6 or SEQ ID No. 8 and have the activities mentioned.

30 The invention furthermore relates to a process for the fermentative preparation of amino acids chosen from the group consisting of L-asparagine, L-threonine, L-serine, L-glutamate, L-glycine, L-alanine, L-cysteine, L-valine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, L-histidine, L-lysine, L-tryptophan and L-

arginine using coryneform bacteria which in particular already produce amino acids and in which the nucleotide sequences which code for the cysD gene, the cysN gene, cysE gene, the cysK gene and/or the cysH gene are enhanced, in particular over-expressed.

The term "enhancement" in this connection describes the increase in the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by increasing the number of copies of the gene or genes, using a potent promoter or using a gene or allele which codes for a corresponding enzyme (protein) having a high activity, and optionally combining these measures.

By enhancement measures, in particular over-expression, the activity or concentration of the corresponding protein is in general increased by at least 10%, 25%, 50%, 75%, 100%, 150%, 200%, 300%, 400% or 500%, up to a maximum of 1000% or 2000%, based on that of the wild-type protein or the activity or concentration of the protein in the starting microorganism.

The microorganisms which the present invention provides can produce L-amino acids from glucose, sucrose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They can be representatives of coryneform bacteria, in particular of the genus *Corynebacterium*. Of the genus *Corynebacterium*, there may be mentioned in particular the species *Corynebacterium glutamicum*, which is known among experts for its ability to produce L-amino acids.

Suitable strains of the genus *Corynebacterium*, in particular of the species *Corynebacterium glutamicum* (*C. glutamicum*), are in particular the known wild-type strains

5 Corynebacterium glutamicum ATCC13032  
Corynebacterium acetoglutamicum ATCC15806  
Corynebacterium acetoacidophilum ATCC13870  
Corynebacterium thermoaminogenes FERM BP-1539  
Corynebacterium melassecola ATCC17965  
Brevibacterium flavum ATCC14067  
Brevibacterium lactofermentum ATCC13869 and  
Brevibacterium divaricatum ATCC14020

10 and L-amino acid-producing mutants or strains prepared therefrom.

The new *cysD*, *cysN*, *cysK*, *cysE* and *cysH* genes of *C. glutamicum* which code for the enzymes sulfate adenylyl transferase (EC 2.7.7.4), cysteine synthase A (EC 4.2.99.8), serine acetyl transferase (EC 2.3.1.30) and 3'-phosphoadenylyl sulfate reductase (EC 1.8.99.4) have been isolated.

To isolate the *cysD* gene, the *cysN* gene, the *cysK* gene, the *cysE* gene, the *cysH* gene or also other genes of *C. glutamicum*, a gene library of this microorganism is first set up in *Escherichia coli* (*E. coli*). The setting up of gene libraries is described in generally known textbooks and handbooks. The textbook by Winnacker: *Gene und Klone, Eine Einführung in die Gentechnologie* (Verlag Chemie, Weinheim, Germany, 1990), or the handbook by Sambrook et al.: *Molecular Cloning, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, 1989) may be mentioned as an example. A well-known gene library is that of the *E. coli* K-12 strain W3110 set up in  $\lambda$  vectors by Kohara et al. (Cell 50, 495 -508 (1987)). Bathe et al. (Molecular and General Genetics, 252:255-265, 1996) describe a gene library of *C. glutamicum* ATCC13032, which was set up with the aid of the cosmid vector SuperCos I (Wahl et al., 1987, Proceedings of the National Academy of Sciences USA, 84:2160-2164) in the *E. coli* K-12 strain NM554 (Raleigh et al., 1988, Nucleic Acids Research 16:1563-1575).

Börmann et al. (Molecular Microbiology 6(3), 317-326 (1992)) in turn describe a gene library of *C. glutamicum* ATCC13032 using the cosmid pH79 (Hohn and Collins, Gene 11, 291-298 (1980)).

- 5 To prepare a gene library of *C. glutamicum* in *E. coli* it is also possible to use plasmids such as pBR322 (Bolivar, Life Sciences, 25, 807-818 (1979)) or pUC9 (Vieira et al., 1982, Gene, 19:259-268). Suitable hosts are, in particular, those *E. coli* strains which are restriction- and recombination-  
10 defective. An example of these is the strain DH5 $\alpha$ mcr, which has been described by Grant et al. (Proceedings of the National Academy of Sciences USA, 87 (1990) 4645-4649). The long DNA fragments cloned with the aid of cosmids can in turn be subcloned in the usual vectors suitable for  
15 sequencing and then sequenced, as is described e.g. by Sanger et al. (Proceedings of the National Academy of Sciences of the United States of America, 74:5463-5467, 1977).

- The resulting DNA sequences can then be investigated with  
20 known algorithms or sequence analysis programs, such as e.g. that of Staden (Nucleic Acids Research 14, 217-232(1986)), that of Marck (Nucleic Acids Research 16, 1829-1836 (1988)) or the GCG program of Butler (Methods of Biochemical Analysis 39, 74-97 (1998)).

- 25 The new DNA sequences of *C. glutamicum* which code for the *cysD*, *cysN*, *cysK*, *cysE* and *cysH* genes and which, as SEQ ID No. 1, SEQ ID No. 4, and SEQ ID No. 7, are constituents of the present invention have been found. The amino acid sequence of the corresponding proteins has furthermore been  
30 derived from the present DNA sequences by the methods described above. The resulting amino acid sequences of the *cysD*, *cysN*, *cysK*, *cysE* and *cysH* gene products are shown in SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 6 and SEQ ID No. 8.

Coding DNA sequences which result from SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7 by the degeneracy of the genetic code are also a constituent of the invention. In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of  
5 SEQ ID No. 1 or SEQ ID No. 4 or parts of SEQ ID No. 4 or SEQ ID No. 7 or parts of SEQ ID No. 7 are a constituent of the invention. Conservative amino acid exchanges, such as e.g. exchange of glycine for alanine or of aspartic acid for glutamic acid in proteins, are furthermore known among  
10 experts as "sense mutations" which do not lead to a fundamental change in the activity of the protein, i.e. are of neutral function. It is furthermore known that changes on the N and/or C terminus of a protein cannot substantially impair or can even stabilize the function  
15 thereof. Information in this context can be found by the expert, inter alia, in Ben-Bassat et al. (Journal of Bacteriology 169:751-757 (1987)), in O'Regan et al. (Gene 77:237-251 (1989)), in Sahin-Toth et al. (Protein Sciences 3:240-247 (1994)), in Hochuli et al. (Bio/Technology  
20 6:1321-1325 (1988)) and in known textbooks of genetics and molecular biology. Amino acid sequences which result in a corresponding manner from SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 6 and SEQ ID No. 8 are also a constituent of the invention.

25 In the same way, DNA sequences which hybridize with SEQ ID No. 1 or parts of SEQ ID No. 1 or SEQ ID No. 4 or parts of SEQ ID No. 4 or SEQ ID No. 7 or parts of SEQ ID No. 7 are a constituent of the invention. Finally, DNA sequences which are prepared by the polymerase chain reaction (PCR) using  
30 primers which result from SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7 are a constituent of the invention. Such oligonucleotides typically have a length of at least 15 nucleotides.

Instructions for identifying DNA sequences by means of  
35 hybridization can be found by the expert, inter alia, in



the handbook "The DIG System Users Guide for Filter Hybridization" from Boehringer Mannheim GmbH (Mannheim, Germany, 1993) and in Liebl et al. (International Journal of Systematic Bacteriology (1991) 41: 255-260). The

5 hybridization takes place under stringent conditions, that is to say only hybrids in which the probe and target sequence, i. e. the polynucleotides treated with the probe, are at least 70% identical are formed. It is known that the stringency of the hybridization, including the washing

10 steps, is influenced or determined by varying the buffer composition, the temperature and the salt concentration. The hybridization reaction is preferably carried out under a relatively low stringency compared with the washing steps (Hybaid Hybridisation Guide, Hybaid Limited, Teddington,

15 UK, 1996).

A 5x SSC buffer at a temperature of approx. 50°C - 68°C, for example, can be employed for the hybridization reaction. Probes can also hybridize here with

20 polynucleotides which are less than 70% identical to the sequence of the probe. Such hybrids are less stable and are removed by washing under stringent conditions. This can be achieved, for example, by lowering the salt concentration to 2x SSC and optionally subsequently 0.5x SSC (The DIG System User's Guide for Filter Hybridisation, Boehringer

25 Mannheim, Mannheim, Germany, 1995) a temperature of approx. 50°C - 68°C being established. It is optionally possible to lower the salt concentration to 0.1x SSC. Polynucleotide fragments which are, for example, at least 70% or at least 80% or at least 90% to 95% identical to the sequence of the

30 probe employed can be isolated by increasing the hybridization temperature stepwise from 50°C to 68°C in steps of approx. 1 - 2°C. Further instructions on hybridization are obtainable on the market in the form of so-called kits (e.g. DIG Easy Hyb from Roche Diagnostics

35 GmbH, Mannheim, Germany, Catalogue No. 1603558).



Instructions for amplification of DNA sequences with the aid of the polymerase chain reaction (PCR) can be found by the expert, inter alia, in the handbook by Gait: Oligonucleotide Synthesis: A Practical Approach (IRL Press, Oxford, UK, 1984) and in Newton and Graham: PCR (Spektrum Akademischer Verlag, Heidelberg, Germany, 1994).

It has been found that coryneform bacteria produce amino acids in an improved manner after over-expression of one or more of the genes chosen from the group consisting of the cysD gene, cysN gene, cysK gene, cysE gene and cysH gene.

To achieve an over-expression, the number of copies of the corresponding genes can be increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene can be mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible promoters, it is additionally possible to increase the expression in the course of fermentative amino acid production. The expression is likewise improved by measures to prolong the life of the mRNA. Furthermore, the enzyme activity is also increased by preventing the degradation of the enzyme protein. The genes or gene constructs can either be present in plasmids with a varying number of copies, or can be integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)), in Eikmanns et al. (Gene 102, 93-98 (1991)), in EP 0 472 869, in US 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991)), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132

(1994)), in LaBarre et al. (Journal of Bacteriology 175, 1001-1007 (1993)), in WO 96/15246, in Malumbres et al. (Gene 134, 15 - 24 (1993)), in JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195  
5 (1998)), in Makrides (Microbiological Reviews 60:512-538 (1996)) and in known textbooks of genetics and molecular biology.

By way of example, for enhancement the *cysD*, *cysN*, *cysK*, *cysE* or *cysH* genes according to the invention were over-  
10 expressed with the aid of episomal plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Numerous known plasmid vectors, such as e.g. pZ1 (Menkel et al., Applied and Environmental Microbiology (1989) 64: 549-554), pEKEx1 (Eikmanns et al., Gene 102:93-  
15 98 (1991)) or pHS2-1 (Sonnen et al., Gene 107:69-74 (1991)) are based on the cryptic plasmids pHM1519, pBL1 or pGA1. Other plasmid vectors, such as e.g. those based on pCG4 (US-A 4,489,160), or pNG2 (Serwold-Davis et al., FEMS Microbiology Letters 66, 119-124 (1990)), or pAG1 (US-A  
20 5,158,891), can be used in the same manner.

Plasmid vectors which are furthermore suitable are also those with the aid of which the process of gene amplification by integration into the chromosome can be used, as has been described, for example, by Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132  
25 (1994)) for duplication or amplification of the *hom-thrB* operon. In this method, the complete gene is cloned in a plasmid vector which can replicate in a host (typically *E. coli*), but not in *C. glutamicum*. Possible vectors are, for example, pSUP301 (Simon et al., Bio/Technology 1, 784-791  
30 (1983)), pK18mob or pK19mob (Schäfer et al., Gene 145, 69-73 (1994)), pGEM-T (Promega Corporation, Madison, WI, USA), pCR2.1-TOPO (Shuman (1994). Journal of Biological Chemistry 269:32678-84; US-A 5,487,993), pCR®Blunt (Invitrogen,  
35 Groningen, Holland; Bernard et al., Journal of Molecular

In addition, it may be advantageous for the production of L-amino acids to enhance, in particular over-express, one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the citric acid cycle, of the pentose phosphate cycle, of amino acid export and optionally regulatory proteins, in addition to the cysD gene, the cysN gene, the cysK gene, the cysE gene and/or the cysH gene.

25 Thus, for the preparation of L-amino acids, in addition to enhancement of the cysD gene, the cysN gene, the cysK gene, the cysE gene and/or the cysH gene, one or more endogene genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- 30 • the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),

- the tpi gene which codes for triose phosphate isomerase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pgk gene which codes for 3-phosphoglycerate kinase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- 5 • the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the pyc gene which codes for pyruvate carboxylase (DE-A-198 31 609),
- 10 • the mgo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the lysC gene which codes for a feed-back resistant aspartate kinase (Accession No.P26512; EP-B-0387527; EP-A-0699759),
- 15 • the lysE gene which codes for lysine export (DE-A-195 48 222),
- the hom gene which codes for homoserine dehydrogenase (EP-A 0131171),
- 20 • the ilvA gene which codes for threonine dehydratase (Möckel et al., Journal of Bacteriology (1992) 8065-8072)) or the ilvA(Fbr) allele which codes for a "feed back resistant" threonine dehydratase (Möckel et al., (1994) Molecular Microbiology 13: 833-842),
- 25 • the ilvBN gene which codes for acetohydroxy-acid synthase (EP-B 0356739),
- the ilvD gene which codes for dihydroxy-acid dehydratase (Sahm and Eggeling (1999) Applied and Environmental Microbiology 65: 1973-1979),

- the *zwa1* gene which codes for the Zwa1 protein (DE: 19959328.0, DSM 13115)

can be enhanced, in particular over-expressed.

5 It may furthermore be advantageous for the production of L-amino acids, in addition to enhancement of the *cysD* gene, the *cysN* gene, the *cysK* gene, the *cysE* gene and/or the *cysH* gene, for one or more genes chosen from the group consisting of

- 10 • the *pck* gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the *pgi* gene which codes for glucose 6-phosphate isomerase (US 09/396,478; DSM 12969),
- the *poxB* gene which codes for pyruvate oxidase (DE: 1995 1975.7; DSM 13114),
- 15 • the *zwa2* gene which codes for the Zwa2 protein (DE: 19959327.2, DSM 13113)

to be attenuated, in particular for the expression thereof to be reduced. For the production of L-cysteine in particular, it may be advantageous, in addition to  
20 enhancement of the *cysD* gene, the *cysN* gene, the *cysK* gene, the *cysE* gene and/or the *cysH* gene, for one or more genes chosen from the group consisting of

- 25 • the *aecD* gene which codes for cystathionine  $\beta$ -lyase (Accession Number M89931 des National Center for Biotechnology Information (NCBI, Bethesda, MD, USA),
- the *metB* gene which codes for cystathione  $\gamma$ -synthase (Accession Number AF1236953 des National Center for Biotechnology Information (NCBI, Bethesda, MD, USA)

to be attenuated, in particular for the expression thereof  
30 to be reduced.

The term "attenuation" in this connection describes the reduction or elimination of the intracellular activity of one or more enzymes (proteins) in a microorganism which are coded by the corresponding DNA, for example by using a weak  
5 promoter or using a gene or allele which codes for a corresponding enzyme with a low activity or inactivates the corresponding gene or enzyme (protein), and optionally combining these measures.

By attenuation measures, the activity or concentration of  
10 the corresponding protein is in general reduced to 0 to 75%, 0 to 50%, 0 to 25%, 0 to 10% or 0 to 5% of the activity or concentration of the wild-type protein or of the activity or concentration of the protein in the starting microorganism.

15 In addition to over-expression of the *cysD* gene, the *cysN* gene, the *cysK* gene, the *cysE* gene and/or the *cysH* gene it may furthermore be advantageous for the production of amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in:  
20 Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982).

The invention also provides the microorganisms prepared according to the invention, and these can be cultured continuously or discontinuously in the batch process (batch  
25 culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for the purpose of production of amino acids. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die  
30 Bioverfahrenstechnik (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).



The culture medium to be used must meet the requirements of the particular strains in a suitable manner. Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General  
5 Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil,  
10 groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture.

15 Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be  
20 used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture.

Phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus.

25 Organic and inorganic sulfur-containing compounds, such as, for example, sulfides, sulfites, sulfates and thiosulfates, can be used as a source of sulfur, in particular for the preparation of sulfur-containing amino acids.

The culture medium must furthermore comprise salts of  
30 metals, such as e. g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances.

Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

- 5 Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia or aqueous ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH of the culture. Antifoams, such as e.g. fatty acid polyglycol esters, can  
10 be employed to control the development of foam. Suitable substances having a selective action, such as e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air,  
15 are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of the desired product has formed. This target is usually reached within 10 hours to 160 hours.
- 20 The fermentation broths obtained in this way, in particular containing L-methionine, usually have a dry weight of 7.5 to 25 wt.% and contain L-methionine. It is furthermore also advantageous if the fermentation is conducted in a sugar-limited procedure at least at the end, but in particular  
25 over at least 30% of the duration of the fermentation. That is to say, the concentration of utilizable sugar in the fermentation medium is reduced to  $\geq 0$  to 3 g/l during this period.

The fermentation broth prepared in this manner, in  
30 particular containing L-methionine, is then further processed. Depending on requirements, the all or some of the biomass can be removed from the fermentation broth by separation methods, such as e.g. centrifugation, filtration, decanting or a combination thereof, or it can  
35 be left completely in this. This broth is then thickened or

concentrated by known methods, such as e.g. with the aid of a rotary evaporator, thin film evaporator, falling film evaporator, by reverse osmosis, or by nanofiltration. This concentrated fermentation broth can then be worked up by  
5 methods of freeze drying, spray drying, spray granulation or by other processes to give a preferably free-flowing, finely divided powder.

This free-flowing, finely divided powder can then in turn be converted by suitable compacting or granulating  
10 processes into a coarse-grained, readily free-flowing, storable and largely dust-free product. In the granulation or compacting it is advantageous to employ conventional organic or inorganic auxiliary substances or carriers, such as starch, gelatin, cellulose derivatives or similar  
15 substances, such as are conventionally used as binders, gelling agents or thickeners in foodstuffs or feedstuffs processing, or further substances, such as, for example, silicas, silicates or stearates.

"Free-flowing" is understood as meaning powders which flow  
20 unimpeded out of the vessel with the opening of 5 mm (millimeters) of a series of glass outflow vessels with outflow openings of various sizes (Klein, Seifen, Öle, Fette, Wachse 94, 12 (1968)).

As described here, "finely divided" means a powder with a  
25 predominant content (> 50 %) with a particle size of 20 to 200  $\mu\text{m}$  diameter. "Coarse-grained" means products with a predominant content (> 50 %) with a particle size of 200 to 2000  $\mu\text{m}$  diameter. In this context, "dust-free" means that the product contains only small contents (< 5 %) with  
30 particle sizes of less than 20  $\mu\text{m}$  diameter. The particle size determination can be carried out with methods of laser diffraction spectrometry. The corresponding methods are described in the textbook on "Teilchengrößenmessung in der Laborpraxis" by R. H. Müller and R. Schuhmann,  
35 Wissenschaftliche Verlagsgesellschaft Stuttgart (1996) or

in the textbook "Introduction to Particle Technology" by M. Rhodes, Verlag Wiley & Sons (1998).

"Storable" in the context of this invention means a product which can be stored for up to 120 days, preferably up to 52 weeks, particularly preferably 60 months, without a substantial loss (< 5%) of methionine occurring.

Alternatively, however, the product can be absorbed on to an organic or inorganic carrier substance which is known and conventional in feedstuffs processing, such as, for example, silicas, silicates, grits, brans, meals, starches, sugars or others, and/or mixed and stabilized with conventional thickeners or binders. Use examples and processes in this context are described in the literature (Die Mühle + Mischfutterschnik 132 (1995) 49, page 817).

Finally, the product can be brought into a state in which it is stable to digestion by animal stomachs, in particular the stomach of ruminants, by coating processes ("coating") using film-forming agents, such as, for example, metal carbonates, silicas, silicates, alginates, stearates, starches, gums and cellulose ethers, as described in DE-C-4100920.

If the biomass is separated off during the process, further inorganic solids, for example added during the fermentation, are in general removed. In addition, the animal feedstuffs additive according to the invention comprises at least the predominant proportion of the further substances, in particular organic substances, which are formed or added and are present in solution in the fermentation broth, where these have not been separated off by suitable processes.

In one aspect of the invention, the biomass can be separated off to the extent of up to 70%, preferably up to 80%, preferably up to 90%, preferably up to 95%, and

particularly preferably up to 100%. In another aspect of the invention, up to 20% of the biomass, preferably up to 15%, preferably up to 10%, preferably up to 5%, particularly preferably no biomass is separated off.

- 5 These organic substances include organic by-products which are optionally produced, in addition to the L-methionine, and optionally discharged by the microorganisms employed in the fermentation. These include L-amino acids chosen from the group consisting of L-lysine, L-valine, L-threonine, L-  
10 alanine or L-tryptophan. They include vitamins chosen from the group consisting of vitamin B1 (thiamine), vitamin B2 (riboflavin), vitamin B5 (pantothenic acid), vitamin B6 (pyridoxine), vitamin B12 (cyanocobalamin), nicotinic acid/nicotinamide and vitamin E (tocopherol). They include  
15 furthermore organic acids which carry one to three carboxyl groups, such as, for example, acetic acid, lactic acid, citric acid, malic acid or fumaric acid. Finally, they also include sugars, such as, for example, trehalose. These compounds are optionally desired if they improve the  
20 nutritional value of the product.

These organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, can also be added, depending on requirements, as a concentrate or pure substance in solid or liquid form during a suitable  
25 process step. These organic substances mentioned can be added individually or as mixtures to the resulting or concentrated fermentation broth, or also during the drying or granulation process. It is likewise possible to add an organic substance or a mixture of several organic  
30 substances to the fermentation broth and a further organic substance or a further mixture of several organic substances during a later process step, for example granulation.

The product described above is suitable as a feedstuffs  
35 additive, i.e. feed additive, for animal nutrition.



The L-methionine content of the animal feedstuffs additive is conventionally 1 wt.% to 80 wt.%, preferably 2 wt.% to 80 wt.%, particularly preferably 4 wt.% to 80 wt.%, and very particularly preferably 8 wt.% to 80 wt.%, based on  
5 the dry weight of the animal feedstuffs additive. Contents of 1 wt.% to 60 wt.%, 2 wt.% to 60 wt.%, 4 wt.% to 60 wt.%, 6 wt.% to 60 wt.%, 1 wt.% to 40 wt.%, 2 wt.% to 40 wt.% or 4 wt.% to 40 wt.% are likewise possible. The water content of the feedstuffs additive is conventionally up to 5 wt.%,  
10 preferably up to 4 wt.%, and particularly preferably less than 2 wt.%.

The invention accordingly also provides a process for the preparation of an L-methionine-containing animal feedstuffs additive from fermentation broths, which comprises the  
15 steps

- a) culture and fermentation of an L-methionine-producing microorganism in a fermentation medium;
  - b) removal of water from the L-methionine-containing fermentation broth (concentration);
  - 20 c) removal of an amount of 0 to 100 wt.% of the biomass formed during the fermentation; and
  - d) drying of the fermentation broth obtained according to a) and/or b) to obtain the animal feedstuffs additive in the desired powder or granule form.
- 25 If desired, one or more of the following steps can furthermore be carried out in the process according to the invention:
- e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic  
30 mixture D,L-methionine, to the products obtained according to a), b) and/or c);



- [ ]
- f) addition of auxiliary substances chosen from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according to a) to d) for stabilization and to increase the storability; or
- 5 g) conversion of the substances obtained according to a) to e) into a form which is stable in an animal stomach, in particular rumen, by coating with film-forming agents.

Methods for the determination of L-amino acids are known  
10 from the prior art. The analysis can thus be carried out, for example, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190) by ion exchange chromatography with subsequent ninhydrin derivation, or it can be carried out by reversed phase HPLC, for example as described by  
15 Lindroth et al. (Analytical Chemistry (1979) 51: 1167-1174).

The process according to the invention is used for fermentative preparation of amino acids.

The following microorganisms were deposited as a pure  
20 culture on 18th May 2001 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

- E. coli DH5 $\alpha$ mcr/pEC-XK99EcysEblex as DSM 14308,
- 25 • E. coli DH5 $\alpha$ mcr/pEC-XK99EcysKalex as DSM 14310,
- E. coli DH5 $\alpha$ mcr/pEC-XK99EcysDalex as DSM 14311,
- E. coli DH5 $\alpha$ mcr/pEC-XK99EcysHalex as DSM 14315.

The present invention is explained in more detail in the following with the aid of embodiment examples.

The isolation of plasmid DNA from *Escherichia coli* and all techniques of restriction, Klenow and alkaline phosphatase treatment were carried out by the method of Sambrook et al. (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY, USA).  
5 Methods for transformation of *Escherichia coli* are also described in this handbook.

The composition of the usual nutrient media, such as LB or TY medium, can also be found in the handbook by Sambrook et al.  
10

#### Example 1

Preparation of a genomic cosmid gene library from  
*Corynebacterium glutamicum* ATCC 13032

Chromosomal DNA from *Corynebacterium glutamicum* ATCC 13032  
15 was isolated as described by Tauch et al. (1995, Plasmid 33:168-179) and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase  
20 (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description  
25 SuperCos1 Cosmid Vector Kit, Code no. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase.

30 The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032

DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of Gigapack II XL Packing  
5 Extract (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217).

For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM MgSO<sub>4</sub> and mixed with an aliquot of the  
10 phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology, 1:190) with 100 mg/l ampicillin. After  
15 incubation overnight at 37°C, recombinant individual clones were selected.

#### Example 2

Isolation and sequencing of the cysD gene, the cysN gene, the cysK gene, the cysE gene or the cysH gene

20 The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product  
25 Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in  
30 the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01), was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 $\alpha$ MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l zeocin.

The plasmid preparation of the recombinant clones was carried out with the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain termination method of Sanger et al. (1977, Proceedings of the National Academy of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig.

The computer-assisted coding region analysis was prepared with the XNIP program (Staden, 1986, Nucleic Acids Research, 14:217-231).

The resulting nucleotide sequences are shown in SEQ ID No. 1 SEQ ID No. 4 and SEQ ID No. 7. Analysis of the nucleotide sequences showed six open reading frames of 915 base pairs, 1302 base pairs, 936 base pairs, 567 base pairs and 786 base pairs, which were called the cysD gene, cysN gene, cysK gene, cysE gene and cysH gene. The cysD gene codes for a protein of 304 amino acids, the cysN gene codes for a protein of 433 amino acids, the cysK gene codes for a protein of 311 amino acids, the cysE gene codes for a protein of 188 amino acids and the cysH gene codes for a protein of 261 amino acids.

### 15 Example 3

Preparation of shuttle expression vectors based on pEC-XK99E for enhancement of the cysD, cysK, cysE and cysH genes in *C. glutamicum*

#### 3.1 Amplification of the cysD, cysK, cysE and cysH genes

20 From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817-1828 (1994)). On the basis of the sequences of the cysD, cysK, cysE and cysH genes known for *C. glutamicum* from Example 2, the following oligonucleotides, listed in Table 1, were  
25 chosen for the polymerase chain reaction (see SEQ ID No. 9, SEQ ID No. 10, SEQ ID No. 11, SEQ ID No. 12, SEQ ID No. 13, SEQ ID No. 14, SEQ ID No. 15 and SEQ ID No. 16). In addition, suitable restriction cleavage sites which allow cloning into the target vector were inserted into the  
30 primers. They are listed in Table 1 and identified by underlining in the nucleotide sequence.

Table 1

| Primer  | Sequence with restriction cleavage site     | Amplified fragment |
|---------|---|--------------------|
| cysDex1 | 5'-ctggtacc-gcggacttcactcatgacca-3'<br>KpnI | cysD<br>(1017 bp)  |
| cysDex2 | 5'-cgtctaga-ggaacctgcggtgcacagac-3'<br>XbaI |                    |
| cysKex1 | 5'-agggtacc-caagcggtcgaccaacaaaa-3'<br>KpnI | cysK<br>(1005 bp)  |
| cysKex2 | 5'-cttctaga-attagtcgcggatgtcttcg-3'<br>XbaI |                    |
| cysEex1 | 5'-ctggtacc-tcacgctgtagacttgacct-3'<br>KpnI | cysE<br>(672 bp)   |
| cysEex2 | 5'-gatctaga-acaacgcactctggagctt-3'<br>XbaI  |                    |
| cysHex1 | 5'-acggtacc-tgagtcgcaacaatgagctt-3'<br>KpnI | cysH<br>(884 bp)   |
| cysHex2 | 5'-gttctaga-cggaggatgtggatggattc-3'<br>XbaI |                    |

The primers shown were synthesized by MWG-Biotech AG (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR Protocols. A Guide to Methods and Applications, 1990, Academic Press) with Pwo-Polymerase from Roche Diagnostics GmbH (Mannheim, Germany). With the aid of the polymerase chain reaction, the primers allow amplification of a DNA fragment 1017 bp in size, which carries the cysD gene, and a DNA fragment 1005 bp in size, which carries the cysK gene, a DNA fragment 672 bp in size, which carries the cysE gene, and a DNA fragment 884 bp in size, which carries the cysH gene. The cysD fragment, the cysK fragment, the cysE fragment and the cysH fragment were cleaved with the restriction



endonucleases KpnI and XbaI and then isolated from the agarose gel with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany).

### 3.2 Construction of the shuttle vector pEC-XK99E

5 The *E. coli* - *C. glutamicum* shuttle vector pEC-XK99E was constructed according to the prior art. The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the  
10 kanamycin resistance gene aph(3')-IIa from *Escherichia coli* (Beck et al. (1982), Gene 19: 327-336), the replication origin of the trc promoter, the termination regions T1 and T2, the lacI<sup>q</sup> gene (repressor of the lac operon of *E. coli*) and a multiple cloning site (mcs) (Norranders, J.M. et al.  
15 Gene 26, 101-106 (1983)) of the plasmid pTRC99A (Amann et al. (1988), Gene 69: 301-315).

The *E. coli* - *C. glutamicum* shuttle vector pEC-XK99E constructed was transferred into *C. glutamicum* DSM5715 by means of electroporation (Liebl et al., 1989, FEMS  
20 Microbiology Letters, 53:299-303). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and  
25 18 g/l Bacto-agar, which had been supplemented with 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology, 144, 915 - 927), cleaved with the restriction endonuclease HindIII, and the plasmid was checked by  
30 subsequent agarose gel electrophoresis.

The plasmid construct thus obtained in this way was called pEC-XK99E and is shown in Figure 1. The strain obtained by electroporation of the plasmid pEC-XK99E in the *C.*

glutamicum strain DSM5715 was called DSM5715/pEC-XK99E and deposited as DSM 13455 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

### 3.3 Cloning of the *cysD*, *cysK*, *cysE* and *cysH* genes in the *E. coli*-*C. glutamicum* shuttle vector pEC-XK99E

The *E. coli* - *C. glutamicum* shuttle vector pEC-XK99E described in Example 3.1 was used as the vector. DNA of this plasmid was cleaved completely with the restriction enzymes KpnI and XbaI and then dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, Product Description SAP, Product No. 1758250).

The fragments *cysD*, approx. 1000 bp in size, *cysK*, approx. 990 bp in size, *cysE*, approx. 660 bp in size and *cysH*, approx. 870 bp in size cleaved with the restriction enzymes KpnI and XbaI and isolated from the agarose gel were in each case mixed with the prepared vector pEC-XK99E and the batches were treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation batches were transformed in the *E. coli* strain DH5 $\alpha$ mcr (Hanahan, In: DNA Cloning. A Practical Approach. Vol. I, IRL-Press, Oxford, Washington DC, USA). Selection of plasmid-carrying cells was made by plating out the transformation batches on LB agar (Lennox, 1955, Virology, 1:190) with 50 mg/l kanamycin. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated from a transformant in each case with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and cleaved with the restriction enzymes KpnI and XbaI to check the plasmid by subsequent agarose gel electrophoresis. The plasmids obtained were called pEC-XK99EcysDalex, pEC-

XK99EcysKalex, pEC-XK99EcysEblex and pEC-XK99EcysHalex.  
They are shown in Figures 2, 3, 4 and 5.

#### Example 4

Transformation of the strain DSM5715 with the plasmids pEC-  
5 XK99EcysDalex, pEC-XK99EcysKalex, pEC-XK99EcysEblex and  
pEC-XK99EcysHalex

The strain DSM5715 was transformed with in each case one of  
the plasmids pEC-XK99EcysDalex, pEC-XK99EcysKalex, pEC-  
XK99EcysEblex and pEC-XK99EcysHalex using the  
10 electroporation method described by Liebl et al., (FEMS  
Microbiology Letters, 53:299-303 (1989)). Selection of the  
transformants took place on LBHIS agar comprising 18.5 g/l  
brain-heart infusion broth, 0.5 M sorbitol, 5 g/l Bacto-  
tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and  
15 18 g/l Bacto-agar, which had been supplemented with 25 mg/l  
kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated from a transformant in each case  
by conventional methods (Peters-Wendisch et al., 1998,  
Microbiology 144, 915-927). DNA of the plasmids pEC-  
20 XK99EcysDalex, pEC-XK99EcysKalex, pEC-XK99EcysEblex and  
pEC-XK99EcysHalex were cleaved with the restriction  
endonucleases KpnI and XbaI. The plasmids were checked by  
subsequent agarose gel electrophoresis. The strains  
obtained were called DSM5715/pEC-XK99EcysDalex,  
25 DSM5715/pEC-XK99EcysKalex, DSM5715/pEC-XK99EcysEblex or  
DSM5715/pEC-XK99EcysHalex.

#### Example 5

##### Preparation of Lysine

The C. glutamicum strains DSM5715/pEC-XK99EcysDalex,  
30 DSM5715/pEC-XK99EcysKalex, DSM5715/pEC-XK99EcysEblex or  
DSM5715/pEC-XK99EcysHalex obtained in Example 4 were  
cultured in a nutrient medium suitable for the production

of lysine and the lysine content in the culture supernatant of each strain was determined.

For this, the strains were first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with  
5 kanamycin (25 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, in each case a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the precultures.

10

## Medium Cg III

NaCl 2.5 g/l

Bacto-Peptide 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Kanamycin (25 mg/l) was added to this. The precultures were incubated for 16 hours at 33°C at 240 rpm on a shaking machine. In each case a main culture was seeded from these precultures such that the initial OD (660nm) of the main  
15 cultures was 0.1. Medium MM was used for the main cultures.

## Medium MM

|   |            |
|---|------------|
| CSL (corn steep liquor)                             | 5 g/l      |
| MOPS (morpholinopropanesulfonic acid)               | 20 g/l     |
| Glucose (autoclaved separately)                     | 50 g/l     |
| <br>(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> | <br>25 g/l |
| KH <sub>2</sub> PO <sub>4</sub>                     | 0.1 g/l    |
| MgSO <sub>4</sub> * 7 H <sub>2</sub> O              | 1.0 g/l    |
| CaCl <sub>2</sub> * 2 H <sub>2</sub> O              | 10 mg/l    |
| FeSO <sub>4</sub> * 7 H <sub>2</sub> O              | 10 mg/l    |
| MnSO <sub>4</sub> * H <sub>2</sub> O                | 5.0mg/l    |
| Biotin (sterile-filtered)                           | 0.3 mg/l   |
| Thiamine * HCl (sterile-filtered)                   | 0.2 mg/l   |
| L-Leucine (sterile-filtered)                        | 0.1 g/l    |
| CaCO <sub>3</sub>                                   | 25 g/l     |

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO<sub>3</sub> autoclaved in the dry state.

Culturing was carried out in a 10 ml volume in a 100 ml conical flask with baffles. Kanamycin (25 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours the OD of the cultures DSM5715, DSM5715/pEC-XK99EcysDalex, DSM5715/pEC-XK99EcysKalex and DSM5715/pEC-

XK99EcysHalex and after 72 hours the OD of the culture  
 DSM5715/pEC-XK99EcysEblex was determined at a measurement  
 wavelength of 660 nm with a Biomek 1000 (Beckmann  
 Instruments GmbH, Munich). The amount of lysine formed was  
 5 in each case determined with an amino acid analyzer from  
 Eppendorf-BioTronik (Hamburg, Germany) by ion exchange  
 chromatography and post-column derivation with ninhydrin  
 detection.

The result of the experiment is shown in Tables 2 and 3.

10

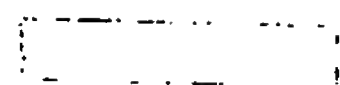
Table 2

| Strain                        | OD (660 nm)<br>(48 h) | Lysine HCl<br>g/l (48 h) |
|-------------------------------|-----------------------|--------------------------|
| DSM5715                       | 11.3                  | 13.11                    |
| DSM5715/pEC-<br>XK99EcysDalex | 13.7                  | 13.54                    |
| DSM5715/pEC-<br>XK99EcysKalex | 13.5                  | 14.35                    |
| DSM5715/pEC-<br>XK99EcysHalex | 11.5                  | 15.22                    |

Table 3

| Strain                        | OD (660 nm)<br>(72 h) | Lysine HCl<br>(72 h) g/l |
|-------------------------------|-----------------------|--------------------------|
| DSM5715                       | 7.17                  | 14.27                    |
| DSM5715/pEC-<br>XK99EcysEblex | 9.0                   | 15.22                    |





# Brief Description of the Figures:

Figure 1: Map of the plasmid pEC-XK99E

Figure 2: Map of the plasmid pEC-XK99EcysDalex

Figure 3: Map of the plasmid pEC-XK99EcysKalex

5 Figure 4: Map of the plasmid pEC-XK99EcysEblex

Figure 5: Map of the plasmid pEC-XK99EcysHalex

The abbreviations and designations used have the following meaning:

|                   |   |
|-------------------|---|
| Kan:              | Kanamycin resistance gene aph(3')-IIa from Escherichia coli       |
| HindIII           | Cleavage site of the restriction enzyme HindIII                   |
| XbaI              | Cleavage site of the restriction enzyme XbaI                      |
| KpnI              | Cleavage site of the restriction enzyme KpnI                      |
| P <sub>trc</sub>  | trc promoter  |
| T1                | Termination region T1   |
| T2                | Termination region T2   |
| per               | Replication effector per  |
| rep               | Replication region rep of the plasmid pGA1                        |
| lacI <sub>q</sub> | lacI <sub>q</sub> repressor of the lac operon of Escherichia coli |
| cysD              | Cloned cysD gene  |
| cysK              | Cloned cysK gene  |
| cysE              | Cloned cysE gene  |
| cysH              | Cloned cysH gene  |

**What is claimed is:**

1. Isolated polynucleotide from coryneform bacteria comprising one or more of the polynucleotide sequences which code for the endogene cysD gene, cysN gene, cysK gene, cysE gene or cysH gene, chosen from the group consisting of
  - a) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 2,
  - b) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 3,
  - c) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 5,
  - d) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 6,
  - e) polynucleotide which is identical to the extent of at least 70% to a polynucleotide which codes for a polypeptide which comprises the amino acid sequence of SEQ ID No. 8,
  - f) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 2,

- g) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 3,
- 5 h) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 5,
- 10 i) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 6,
- 15 j) polynucleotide which codes for a polypeptide which comprises an amino acid sequence which is identical to the extent of at least 70% to the amino acid sequence of SEQ ID No. 8,
- k) polynucleotide which is complementary to the polynucleotides of a), b), c), d), e), f), g), h), i), or j), and
- 20 l) polynucleotide comprising at least 15 successive nucleotides of the polynucleotide sequence of a), b), c), d), e), f), g), h), i), j) or k),
- the polypeptides preferably having the corresponding activities, namely of sulfate adenylyltransferase, cysteine synthase A, serine acetyltransferase or 3'-phosphoadenylyl sulfate reductase.
- 25
2. Polynucleotide according to claim 1, wherein the polynucleotide is a preferably recombinant DNA which is capable of replication in coryneform bacteria.
- 30 3. Polynucleotide according to claim 1, wherein the polynucleotide is an RNA.

4. Polynucleotide according to claim 2, comprising the nucleic acid sequence as shown in SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7.
5. DNA according to claim 2 which is capable of replication, comprising
  - (i) the nucleotide sequence shown in SEQ ID No. 1, SEQ ID No. 4 or SEQ ID No. 7, or
  - (ii) at least one sequence which corresponds to sequence (i) within the range of the degeneration of the genetic code, or
  - (iii) at least one sequence which hybridizes with the sequence complementary to sequence (i) or (ii), and optionally
  - (iv) sense mutations of neutral function in (i).
6. DNA according to claim 5 which is capable of replication, characterized in that the hybridization is carried out under a stringency corresponding to at most 2x SSC.
7. Polynucleotide sequence according to claim 1, which codes for a polypeptide which comprises the amino acid sequence shown in SEQ ID No. 2, SEQ ID No. 3, SEQ ID No. 5, SEQ ID No. 6 or SEQ ID No. 8.
8. A coryneform bacterium in which the cysD gene, cysN gene, cysK gene, cysE gene and/or the cysH gene are enhanced, in particular over-expressed.
9. DSM 14308 deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen [German Collection of Microorganisms and Cell Cultures], Braunschweig, Germany).

10. Escherichia coli strain DH5 $\alpha$ mcr/pEC-XK99EcysKalex as  
DSM 14310 deposited at the Deutsche Sammlung für  
Mikroorganismen und Zellkulturen [German Collection of  
Microorganisms and Cell Cultures], Braunschweig,  
5 Germany.
11. Escherichia coli strain DH5 $\alpha$ mcr/pEC-XK99EcysDalex as  
DSM 14311 deposited at the Deutsche Sammlung für  
Mikroorganismen und Zellkulturen [German Collection of  
Microorganisms and Cell Cultures], Braunschweig,  
10 Germany.
12. Escherichia coli strain DH5 $\alpha$ mcr/pEC-XK99EcysHalex as  
DSM 14315 deposited at the Deutsche Sammlung für  
Mikroorganismen und Zellkulturen [German Collection of  
Microorganisms and Cell Cultures], Braunschweig,  
15 Germany.
13. Process for the fermentative preparation of L-amino  
acids, in particular L-lysine, L-cysteine and L-  
methionine, characterized in that the following steps  
are carried out:
- 20 a) fermentation of the coryneform bacteria which  
produce the desired L-amino acid and in which at  
least the cysD gene, cysN gene, cysK gene, cysE  
gene and/or the cysH gene or nucleotide sequences  
which code for them is or are enhanced, in  
25 particular over-expressed;
- b) concentration of the L-amino acid in the medium or  
in the cells of the bacteria, and
- c) isolation of the L-amino acid.
14. Process according to claim 13, characterized in that  
30 bacteria in which further genes of the biosynthesis  
pathway of the desired L-amino acid are additionally  
enhanced are employed.

15. Process according to claim 13, characterized in that bacteria in which the metabolic pathways which reduce the formation of the desired L-amino acid are at least partly eliminated are employed.
- 5 16. Process according to claim 13, characterized in that a strain transformed with a plasmid vector is employed, and the plasmid vector carries the nucleotide sequence which codes for the cysD gene, cysN gene, cysK gene, cysE gene and/or cysH gene.
- 10 17. Process according to claim 13, characterized in that the expression of the polynucleotides which code for the cysD gene, cysN gene, cysK gene, cysE gene and/or cysH gene is enhanced, in particular over-expressed.
- 15 18. Process according to claim 13, characterized in that the catalytic properties of the polypeptides (enzyme proteins) for which the polynucleotides cysD, cysN, cysK, cysE and/or cysH code are increased.
- 20 19. Process according to claim 13, characterized in that for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the endogene genes chosen from the group consisting of
- 19.1 the dapA gene which codes for dihydrodipicolinate synthase,
- 25 19.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
- 19.3 the tpi gene which codes for triose phosphate isomerase,
- 30 19.4 the pgk gene which codes for 3-phosphoglycerate kinase,



- 19.5 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 19.6 the pyc gene which codes for pyruvate carboxylase,
- 5 19.7 the mgo gene which codes for malate-quinone oxidoreductase,
- 19.8 the lysC gene which codes for a feed-back resistant aspartate kinase,
- 19.9 the lysE gene which codes for lysine export,
- 10 19.10 the hom gene which codes for homoserine dehydrogenase
- 19.11 the ilvA gene which codes for threonine dehydratase or the ilvA(Fbr) allele which codes for a feed back resistant threonine dehydratase,
- 15 19.12 the ilvBN gene which codes for acetohydroxy-acid synthase,
- 19.13 the ilvD gene which codes for dihydroxy-acid dehydratase,
- 20 19.14 the zwal gene which codes for the Zwal protein, is or are enhanced or over-expressed are fermented.
20. Process according to claim 13, characterized in that for the preparation of L-amino acids, coryneform microorganisms in which at the same time one or more of the genes chosen from the group consisting of
- 25 20.1 the pck gene which codes for phosphoenol pyruvate carboxykinase,

- 20.2 the *pgi* gene which codes for glucose 6-phosphate isomerase,
- 20.3 the *poxB* gene which codes for pyruvate oxidase,
- 20.4 the *zwa2* gene which codes for the Zwa2 protein,
- 5 is or are attenuated are fermented.
21. Process according to claim 13, characterized in that for the preparation of L-cysteine, in addition to enhancement of the *cysD* gene, the *cysN* gene, the *cysK* gene, the *cysE* gene and/or the *cysH* gene, one or more
- 10 genes chosen from the group consisting of
- 21.1 the *aecD* gene which codes for cystathionine  $\beta$ -lyase,
- 21.2 the *metB* gene which codes for cystathionine  $\gamma$ -synthase [sic],
- 15 is or are attenuated, in particular reduced in expression.
22. Coryneform bacteria which contain a vector which carries a polynucleotide according to claim 1.
23. Process according to one or more of claims 13-21,
- 20 characterized in that microorganisms of the species *Corynebacterium glutamicum* are employed.
24. Process according to claim 23, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysDalex is employed.
- 25 25. Process according to claim 23, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysKalex is employed.

26. Process according to claim 23, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysEblex is employed.
27. Process according to claim 23, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysHalex is employed.
28. Process for the preparation of an L-methionine-containing animal feedstuffs additive from fermentation broths, characterized by the steps
- a) culture and fermentation of an L-methionine-producing microorganism in a fermentation medium;
  - b) removal of water from the L-methionine-containing fermentation broth (concentration);
  - c) removal of an amount of 0 to 100 wt.% of the biomass formed during the fermentation; and
  - d) drying of the fermentation broth obtained according to b) and/or c) to obtain the animal feedstuffs additive in the desired powder or granule form.
29. Process according to claim 28, characterized in that microorganisms in which further genes of the biosynthesis pathway of L-methionine are additionally enhanced are employed.
30. Process according to claim 28, characterized in that microorganisms in which the metabolic pathways which reduce the formation of L-methionine are at least partly eliminated are employed.
31. Process according to claim 28, characterized in that the expression of the polynucleotides which code for the cysD, cysN, cysK, cysE or cysH gene is enhanced, in particular over-expressed.

32. Process according to one or more of claims 28-31, characterized in that microorganisms of the species *Corynebacterium glutamicum* are employed.
- 5 33. Process according to claim 32, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysDalex is employed.
34. Process according to claim 32, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysKalex is employed.
- 10 35. Process according to claim 32, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysEblex is employed.
- 15 36. Process according to claim 32, characterized in that the *Corynebacterium glutamicum* strain DSM5715/pEC-XK99EcysHalex is employed.
37. Process according to claim 28, characterized in that one or more of the following steps is or are additionally also carried out:
- 20 e) addition of one or more organic substances, including L-methionine and/or D-methionine and/or the racemic mixture D,L-methionine, to the products obtained according to b), c) and/or d);
- 25 f) addition of auxiliary substances chosen from the group consisting of silicas, silicates, stearates, grits and bran to the substances obtained according to b) to e) for stabilization and to increase the storability; or
- 30 g) conversion of the substances obtained according to b) to f) into a form which is stable in an animal stomach, in particular rumen, by coating with film-forming agents.

38. Process according to claim 28 or 37, characterized in that some of the biomass is removed.
39. Process according to claim 38, characterized in that up to 100% of the biomass is removed.
- 5 40. Process according to claim 28 or 37, characterized in that the water content is up to 5 wt.%.
41. Process according to claim 40, characterized in that the water content is less than 2 wt.%.
- 10 42. Process according to claim 37, 38, 39, 40 or 41, characterized in that the film-forming agents are metal carbonates, silicas, silicates, alginates, stearates, starches, gums or cellulose ethers.
43. Animal feedstuffs additive prepared according to claims 28 to 42.
- 15 44. Animal feedstuffs additive according to claim 43, which comprises 1 wt.% to 80 wt.% L\_methionine, D-methionine, D,L-methionine or a mixture thereof, based on the dry weight of the animal feedstuffs additive.
- 20 45. Process for discovering RNA, cDNA and DNA in order to isolate nucleic acids, or polynucleotides or genes which code for sulfate adenylyl transferase, cysteine synthase A, serine acetyl transferase and/or 3'-phosphoadenylyl sulfate reductase or have a high similarity with the sequences of the cysD gene, the cysN gene, the cysK gene, the cysE gene and/or the cysH gene, characterized in that the polynucleotide comprising the polynucleotide sequences according to claims 1, 2, 3 or 4 is employed as hybridization probes.
- 25

Figure 1: Plasmid pEC-XK99E

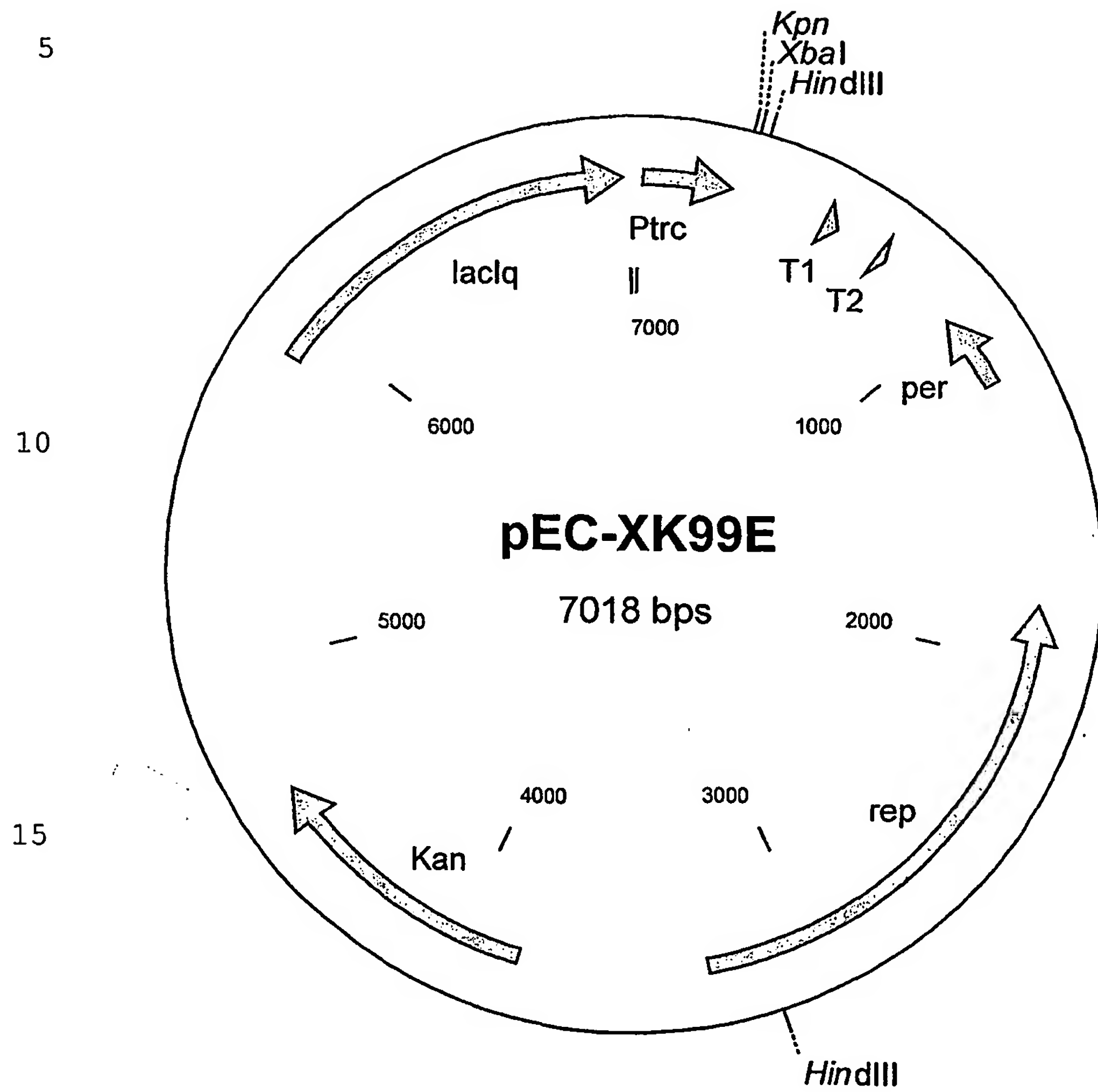




Figure 2: Plasmid pEC-XK99EcysDa1ex

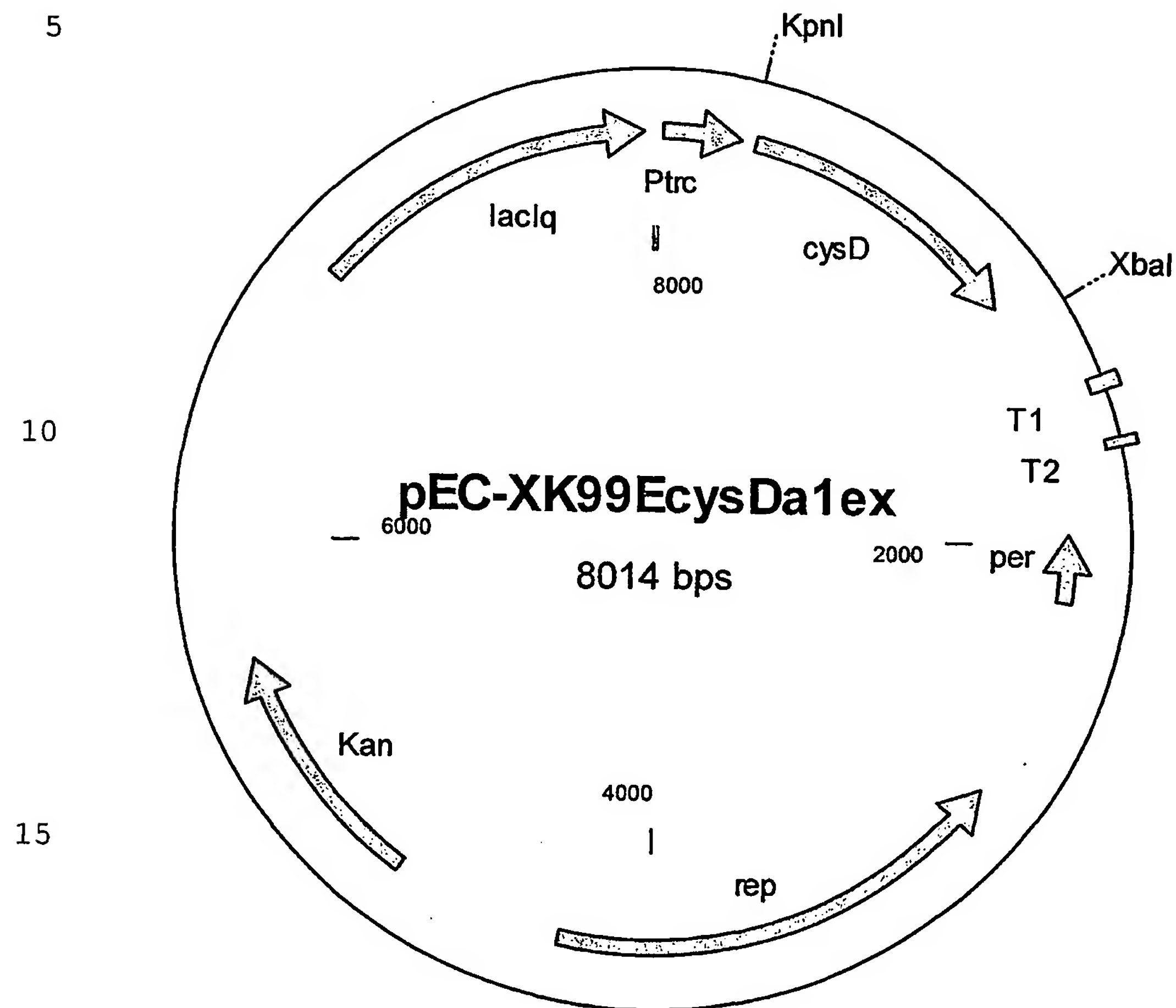
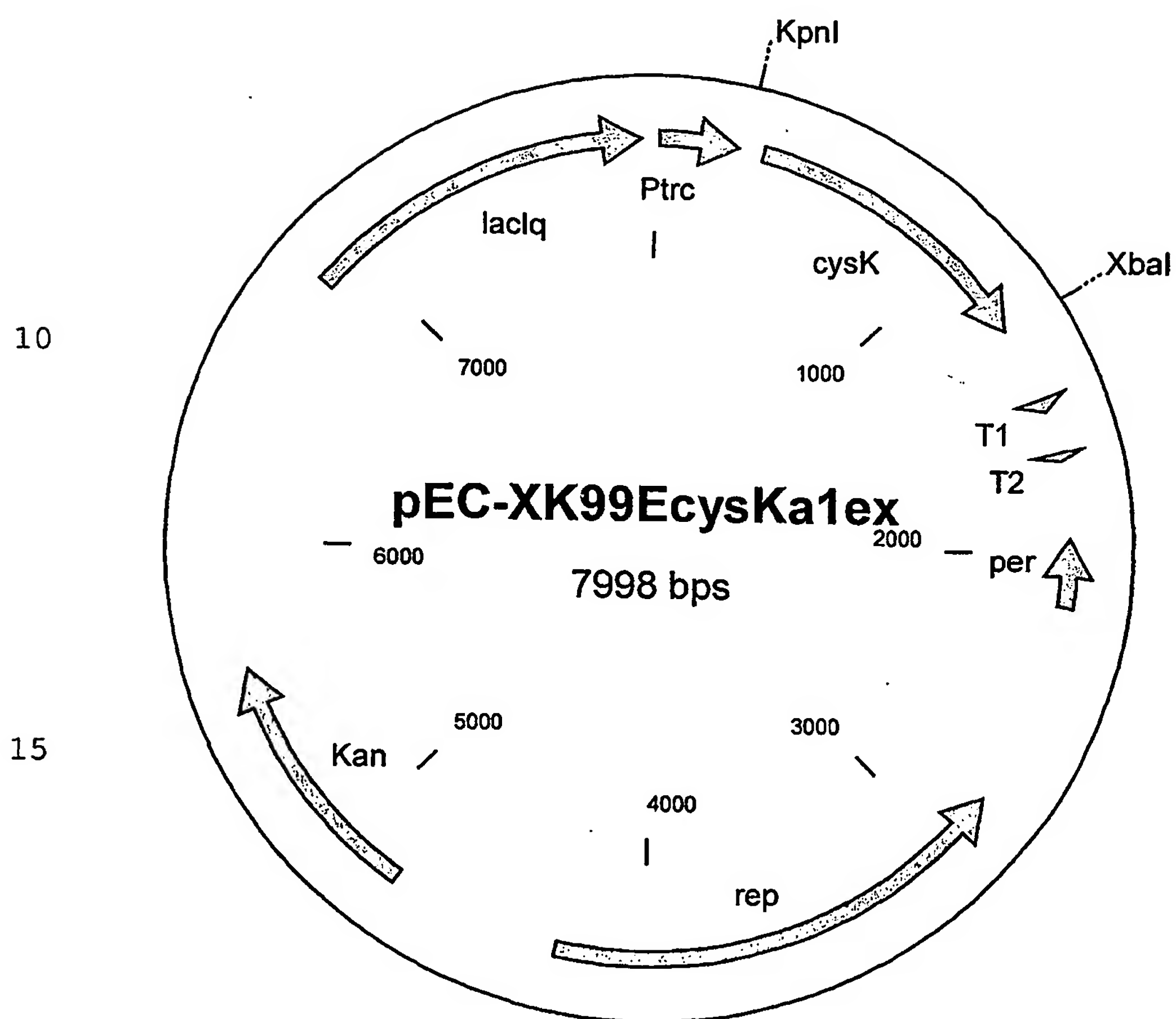


Figure 3: Plasmid pEX-XK99EcysKa1ex

5



15

20

Figure 4: Plasmid pEC-XK99EcysEb1ex

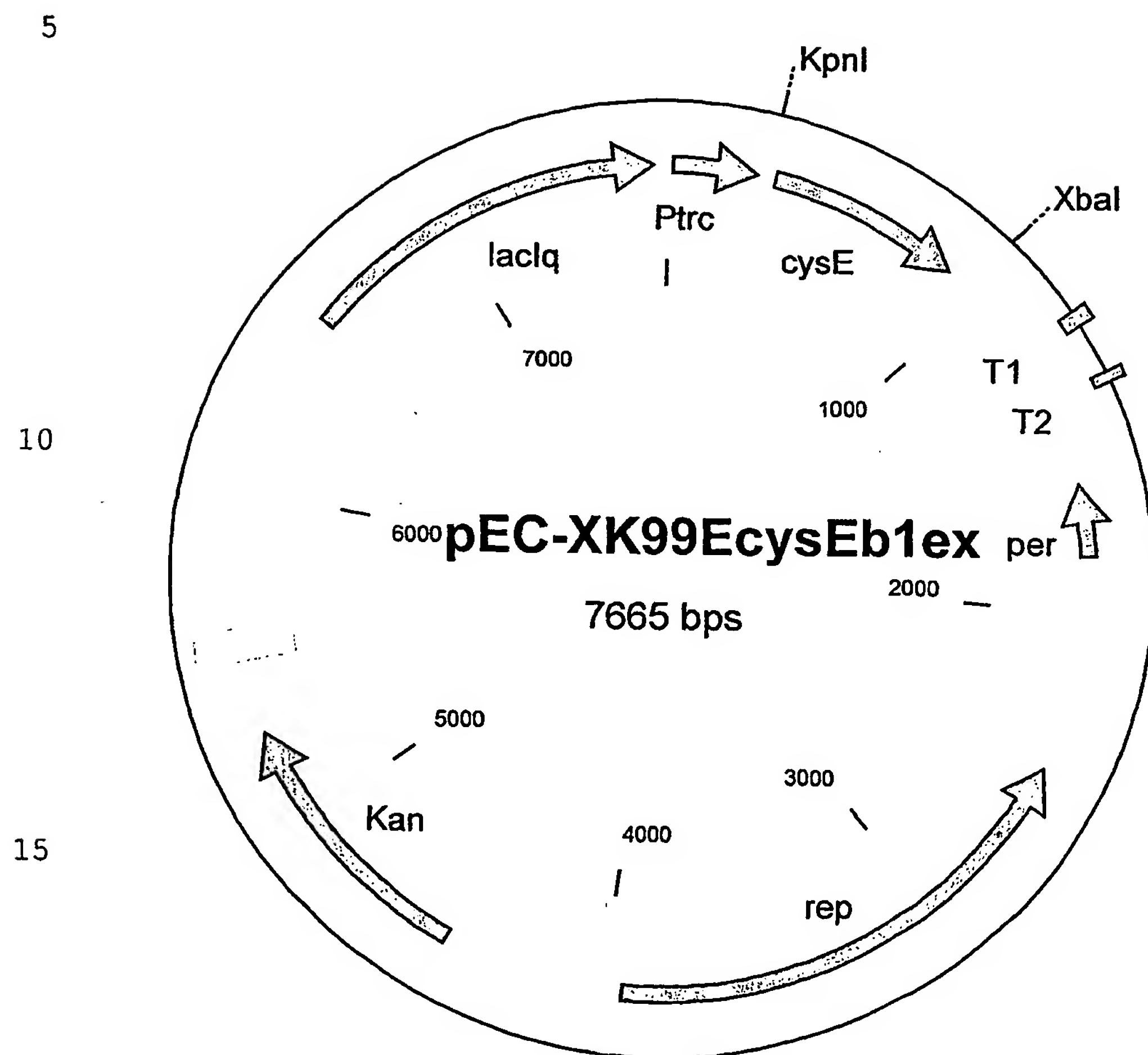
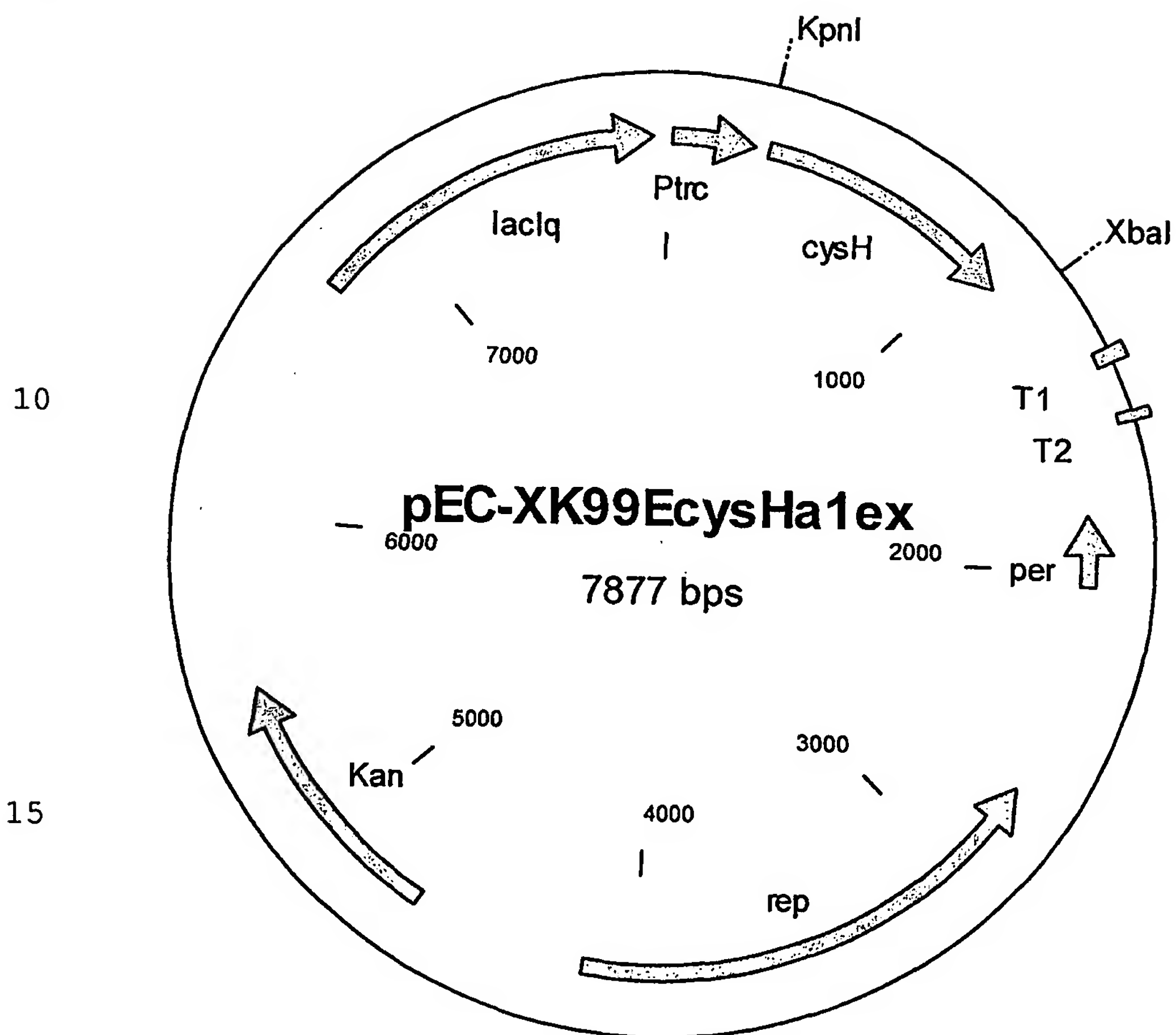


Figure 5: Plasmid pEC-XKcysHalex

5



10

15

20

## SEQUENCE PROTOCOL

&lt;110&gt; Degussa AG

5 <120> Nucleotide sequences which code for the  
cysD, cysN, cysK, cysE and cysH genes

&lt;130&gt; 000491 BT

10 <140>  
<141>

&lt;160&gt; 16

15 &lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 2640

&lt;212&gt; DNA

20 &lt;213&gt; Corynebacterium glutamicum

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (232)..(1143)

25 &lt;223&gt; cysD gene

&lt;220&gt;

&lt;221&gt; CDS

&lt;222&gt; (1146)..(2444)

30 &lt;223&gt; cysN gene

&lt;400&gt; 1

tgcgctgagc ttggatgcca ccggcaggct caagatttct ccaattatca cctggtcatt 60  
 35 ggaggaaacc aacgagttca ttgcggacaa caacctcatc gatcaccac ttacccatca 120  
 gggttatcca tcaattggat gcgaaacctg cacccttcct gttgctgaag gacaagaccc 180  
 tagggccggc cgttgggctg gaaacgcaa gacagaatgc ggacttcact c atg acc 237  
 40 Met Thr  
 1

aca acc gtt gca tca gta cta tcc cca cac ctt aaa gat ctt gaa aat 285  
 Thr Thr Val Ala Ser Val Leu Ser Pro His Leu Lys Asp Leu Glu Asn  
 45 5 10 15

gaa tcc atc cac atc ctc cgc gag gta gct ggc cag ttt gat aag gtc 333  
 Glu Ser Ile His Ile Leu Arg Glu Val Ala Gly Gln Phe Asp Lys Val  
 20 25 30

50 ggc ctg ctg ttt tcc ggc ggt aag gat tcc gtc gtg gtg tac gag ctt 381  
 Gly Leu Leu Phe Ser Gly Gly Lys Asp Ser Val Val Val Tyr Glu Leu  
 35 40 45 50

55 gcg cgc cgc gct ttc gct cca gct aac gtg cct ttt gaa ttg ctg cac 429  
 Ala Arg Arg Ala Phe Ala Pro Ala Asn Val Pro Phe Glu Leu Leu His  
 55 60 65

|    |   |      |
|----|---|------|
|    | gtg gac acc ggc cac aac ttc cca gag gtt ttg gaa ttc cgc gac aac | 477  |
|    | Val Asp Thr Gly His Asn Phe Pro Glu Val Leu Glu Phe Arg Asp Asn |      |
|    | 70 75 80  |      |
| 5  | ctg gtg gag cgc acc ggc gcc cgc ctg cgc gta gct aaa gtc cag gac | 525  |
|    | Leu Val Glu Arg Thr Gly Ala Arg Leu Arg Val Ala Lys Val Gln Asp |      |
|    | 85 90 95  |      |
| 10 | tgg atc gat cgc ggt gac ctg cag gaa cgc cca gac ggc acc cgc aac | 573  |
|    | Trp Ile Asp Arg Gly Asp Leu Gln Glu Arg Pro Asp Gly Thr Arg Asn |      |
|    | 100 105 110   |      |
| 15 | cca ctg cag act gtc cct ttg gtg gag acc atc gct gag cag ggc tac | 621  |
|    | Pro Leu Gln Thr Val Pro Leu Val Glu Thr Ile Ala Glu Gln Gly Tyr |      |
|    | 115 120 125 130   |      |
| 20 | gac gca gtg ctt ggt ggc gct cgc cgc gat gag gag cgt gcc cgc gcc | 669  |
|    | Asp Ala Val Leu Gly Gly Ala Arg Arg Asp Glu Glu Arg Ala Arg Ala |      |
|    | 135 140 145   |      |
|    | aag gag cgt gtg ttc tct gtg cgt gac tcc ttc ggt ggt tgg gat cca | 717  |
|    | Lys Glu Arg Val Phe Ser Val Arg Asp Ser Phe Gly Gly Trp Asp Pro |      |
|    | 150 155 160   |      |
| 25 | cgc cgt cag cgc cca gag ctg tgg acc ctc tac aac ggt ggc cac ctg | 765  |
|    | Arg Arg Gln Arg Pro Glu Leu Trp Thr Leu Tyr Asn Gly Gly His Leu |      |
|    | 165 170 175   |      |
| 30 | cca ggc gaa aac atc cgt gtt ttc cca atc tcc aac tgg act gaa gct | 813  |
|    | Pro Gly Glu Asn Ile Arg Val Phe Pro Ile Ser Asn Trp Thr Glu Ala |      |
|    | 180 185 190   |      |
| 35 | gac att tgg gag tac atc ggc gcc cgt ggc atc gaa ctt cca ccg atc | 861  |
|    | Asp Ile Trp Glu Tyr Ile Gly Ala Arg Gly Ile Glu Leu Pro Pro Ile |      |
|    | 195 200 205 210   |      |
| 40 | tac ttc tcc cac gac cgc gaa gtt ttc gag cgc gac ggc atg tgg ctg | 909  |
|    | Tyr Phe Ser His Asp Arg Glu Val Phe Glu Arg Asp Gly Met Trp Leu |      |
|    | 215 220 225   |      |
|    | acc gca ggc gag tgg ggt gga cca aag aag ggc gag gag atc gtc acc | 957  |
|    | Thr Ala Gly Glu Trp Gly Gly Pro Lys Lys Gly Glu Glu Ile Val Thr |      |
|    | 230 235 240   |      |
| 45 | aag act gtc cgc tac cgc acc gtc ggc gat atg tcc tgc acc ggt gct | 1005 |
|    | Lys Thr Val Arg Tyr Arg Thr Val Gly Asp Met Ser Cys Thr Gly Ala |      |
|    | 245 250 255   |      |
| 50 | gtg ctc tcc gaa gcc cgc acc att gac gat gtg atc gaa gag atc gcc | 1053 |
|    | Val Leu Ser Glu Ala Arg Thr Ile Asp Asp Val Ile Glu Glu Ile Ala |      |
|    | 260 265 270   |      |
| 55 | acc tcc acc ctt acc gaa cgt ggc gca acc cgc gcc gat gac cgc ctc | 1101 |
|    | Thr Ser Thr Leu Thr Glu Arg Gly Ala Thr Arg Ala Asp Asp Arg Leu |      |
|    | 275 280 285 290   |      |
|    | agc gaa tcc gca atg gaa gac cgc aag aag gaa ggc tac ttc tg atg  | 1148 |
|    | Ser Glu Ser Ala Met Glu Asp Arg Lys Lys Glu Gly Tyr Phe Met     |      |
|    | 295 300 305   |      |



|    |   |      |
|----|---|------|
| 5  | act gct cca acc ttg aat aaa gca tcc gaa aag att gca tca cgc gag | 1196 |
|    | Thr Ala Pro Thr Leu Asn Lys Ala Ser Glu Lys Ile Ala Ser Arg Glu |      |
|    | 310 315 320   |      |
| 10 | acc ctt cgt ctg tgc acc gca ggt tcc gta gat gat ggc aag tcc acc | 1244 |
|    | Thr Leu Arg Leu Cys Thr Ala Gly Ser Val Asp Asp Gly Lys Ser Thr |      |
|    | 325 330 335   |      |
| 15 | ttc gtc ggc cgc ctc ctg cac gac acc aag tct gtt ctt gct gat cag | 1292 |
|    | Phe Val Gly Arg Leu Leu His Asp Thr Lys Ser Val Leu Ala Asp Gln |      |
|    | 340 345 350   |      |
| 20 | ctg gct tcc gta gag cgc acc tcc gcc gac cgt ggc ttc gaa ggc ctc | 1340 |
|    | Leu Ala Ser Val Glu Arg Thr Ser Ala Asp Arg Gly Phe Glu Gly Leu |      |
|    | 355 360 365   |      |
| 25 | gac ctg tca ctc ctc gtc gac ggc ctg cgc gcc gag cgt gag cag ggc | 1388 |
|    | Asp Leu Ser Leu Leu Val Asp Gly Leu Arg Ala Glu Arg Glu Gln Gly |      |
|    | 370 375 380 385   |      |
| 30 | atc acc atc gac gtt gcc tac cgc tac ttc gcc acc gac aag cgc acc | 1436 |
|    | Ile Thr Ile Asp Val Ala Tyr Arg Tyr Phe Ala Thr Asp Lys Arg Thr |      |
|    | 390 395 400   |      |
| 35 | ttc atc ctg gct gat acc cca ggt cac gtg cag tac acc cgc aac acc | 1484 |
|    | Phe Ile Leu Ala Asp Thr Pro Gly His Val Gln Tyr Thr Arg Asn Thr |      |
|    | 405 410 415   |      |
| 40 | gtc acc ggc gtc tcc acc tcc cag gtt gta gtt ttg ctt gtc gac gcc | 1532 |
|    | Val Thr Gly Val Ser Thr Ser Gln Val Val Val Leu Leu Val Asp Ala |      |
|    | 420 425 430   |      |
| 45 | cgc cac ggc gtc gtc gag cag acc cgc cgc cac ctg tcc gta tcg gct | 1580 |
|    | Arg His Gly Val Val Glu Gln Thr Arg Arg His Leu Ser Val Ser Ala |      |
|    | 435 440 445   |      |
| 50 | ctg ctg ggc gta cgc acg gtg atc ctc gca gtc aac aaa att gac ctt | 1628 |
|    | Leu Leu Gly Val Arg Thr Val Ile Leu Ala Val Asn Lys Ile Asp Leu |      |
|    | 450 455 460 465   |      |
| 55 | gtt gat tac agc gaa gaa gtc ttc cgc aac att gaa aag gaa ttc gtt | 1676 |
|    | Val Asp Tyr Ser Glu Glu Val Phe Arg Asn Ile Glu Lys Glu Phe Val |      |
|    | 470 475 480   |      |
| 60 | ggc ctg gca tct gca ctt gat gtc aca gac acc cac gtt gtt cca atc | 1724 |
|    | Gly Leu Ala Ser Ala Leu Asp Val Thr Asp Thr His Val Val Pro Ile |      |
|    | 485 490 495   |      |
| 65 | tct gcg ctc aag ggc gac aac gtt gca gaa cct tcc acc cac atg gat | 1772 |
|    | Ser Ala Leu Lys Gly Asp Asn Val Ala Glu Pro Ser Thr His Met Asp |      |
|    | 500 505 510   |      |
| 70 | tgg tac acc gga cca acc gtg ctg gaa atc ctg gaa aac gta gaa gtt | 1820 |
|    | Trp Tyr Thr Gly Pro Thr Val Leu Glu Ile Leu Glu Asn Val Glu Val |      |
|    | 515 520 525   |      |

|    |   |      |
|----|---|------|
|    | tcc cac ggc cgt gca cac gac ctg ggc ttc cgc ttc cca atc cag tac     | 1868 |
|    | Ser His Gly Arg Ala His Asp Leu Gly Phe Arg Phe Pro Ile Gln Tyr     |      |
|    | 530 535 540 545   |      |
| 5  | gtc atc cgc gag cac gcc acc gac tac cgt ggc tac gcc ggc acc atc     | 1916 |
|    | Val Ile Arg Glu His Ala Thr Asp Tyr Arg Gly Tyr Ala Gly Thr Ile     |      |
|    | 550 555 560   |      |
| 10 | aac gct ggt tcc gtc tcc gtg ggc gat acc gtg tac cta cct gaa ggc     | 1964 |
|    | Asn Ala Gly Ser Val Ser Val Gly Asp Thr Val Tyr Leu Pro Glu Gly     |      |
|    | 565 570 575   |      |
| 15 | cgc acc acc cag gtc acc cac atc gat tcc gct gac gga tcc ctc cag     | 2012 |
|    | Arg Thr Thr Gln Val Thr His Ile Asp Ser Ala Asp Gly Ser Leu Gln     |      |
|    | 580 585 590   |      |
| 20 | acc gca tca gtt gga gaa gcc gtt gtc ctg cgc cta gcc cag gaa atc     | 2060 |
|    | Thr Ala Ser Val Gly Glu Ala Val Val Leu Arg Leu Ala Gln Glu Ile     |      |
|    | 595 600 605   |      |
|    | gac ctc atc cgc ggc gaa ctc atc gct ggc gaa gac cgc cca gaa tcc     | 2108 |
|    | Asp Leu Ile Arg Gly Glu Leu Ile Ala Gly Glu Asp Arg Pro Glu Ser     |      |
|    | 610 615 620 625   |      |
| 25 | gtt cgc tcc ttc aac gcc act gtt gtt ggc ttg gcc gat cgc acc atc     | 2156 |
|    | Val Arg Ser Phe Asn Ala Thr Val Val Gly Leu Ala Asp Arg Thr Ile     |      |
|    | 630 635 640   |      |
| 30 | aaa cca ggt gca gca gtc aag gtt cgc tac ggc acc gag ctg gtc cgc     | 2204 |
|    | Lys Pro Gly Ala Ala Val Lys Val Arg Tyr Gly Thr Glu Leu Val Arg     |      |
|    | 645 650 655   |      |
| 35 | gga cgc gtc gca gcc atc gaa cga gtc ctc gac atc gac ggc gtc aac     | 2252 |
|    | Gly Arg Val Ala Ala Ile Glu Arg Val Leu Asp Ile Asp Gly Val Asn     |      |
|    | 660 665 670   |      |
| 40 | gac aac gaa gca cca gaa acc tac ggc ctc aac gac atc gca cac gtg     | 2300 |
|    | Asp Asn Glu Ala Pro Glu Thr Tyr Gly Leu Asn Asp Ile Ala His Val     |      |
|    | 675 680 685   |      |
|    | cgc atc gac gtt gca ggc gaa ctc gaa gtt gaa gat tac gct gcc cgc     | 2348 |
|    | Arg Ile Asp Val Ala Gly Glu Leu Glu Val Glu Asp Tyr Ala Ala Arg     |      |
|    | 690 695 700 705   |      |
| 45 | ggc gcc atc gga tcc ttc ctc ctc atc gac caa tcc tcc ggc gat acc     | 2396 |
|    | Gly Ala Ile Gly Ser Phe Leu Leu Ile Asp Gln Ser Ser Gly Asp Thr     |      |
|    | 710 715 720   |      |
| 50 | ctc gca gct ggc ttg gtt ggc cac cgc cta cgc aat aac tgg tcg atc     | 2444 |
|    | Leu Ala Ala Gly Leu Val Gly His Arg Leu Arg Asn Asn Trp Ser Ile     |      |
|    | 725 730 735   |      |
|    | tagaccagtg tcttaggcaa gacccattt aggacacctc atgattcccc tgattacgct    | 2504 |
| 55 | ttcccacggt tcccgcacaaa agtccgcagc tgcaggcatt actgcgctga ctcattgaggc | 2564 |
|    | cggacgaatg ctggaaacac cagccgtgga agcgcatttta gagcttgctg aaccttcctt  | 2624 |
|    | tgatcaggtt gtggca   | 2640 |

5 <210> 2  
 <211> 304  
 <212> PRT  
 <213> Corynebacterium glutamicum

10 <400> 2  
 Met Thr Thr Thr Val Ala Ser Val Leu Ser Pro His Leu Lys Asp Leu  
 1 5 10 15  
 Glu Asn Glu Ser Ile His Ile Leu Arg Glu Val Ala Gly Gln Phe Asp  
 20 25 30  
 Lys Val Gly Leu Leu Phe Ser Gly Gly Lys Asp Ser Val Val Val Tyr  
 35 40 45  
 15 Glu Leu Ala Arg Arg Ala Phe Ala Pro Ala Asn Val Pro Phe Glu Leu  
 50 55 60  
 Leu His Val Asp Thr Gly His Asn Phe Pro Glu Val Leu Glu Phe Arg  
 65 70 75 80  
 20 Asp Asn Leu Val Glu Arg Thr Gly Ala Arg Leu Arg Val Ala Lys Val  
 85 90 95  
 Gln Asp Trp Ile Asp Arg Gly Asp Leu Gln Glu Arg Pro Asp Gly Thr  
 100 105 110  
 Arg Asn Pro Leu Gln Thr Val Pro Leu Val Glu Thr Ile Ala Glu Gln  
 115 120 125  
 25 Gly Tyr Asp Ala Val Leu Gly Gly Ala Arg Arg Asp Glu Glu Arg Ala  
 130 135 140  
 Arg Ala Lys Glu Arg Val Phe Ser Val Arg Asp Ser Phe Gly Gly Trp  
 145 150 155 160  
 30 Asp Pro Arg Arg Gln Arg Pro Glu Leu Trp Thr Leu Tyr Asn Gly Gly  
 165 170 175  
 His Leu Pro Gly Glu Asn Ile Arg Val Phe Pro Ile Ser Asn Trp Thr  
 180 185 190  
 Glu Ala Asp Ile Trp Glu Tyr Ile Gly Ala Arg Gly Ile Glu Leu Pro  
 195 200 205  
 35 Pro Ile Tyr Phe Ser His Asp Arg Glu Val Phe Glu Arg Asp Gly Met  
 210 215 220  
 Trp Leu Thr Ala Gly Glu Trp Gly Gly Pro Lys Lys Gly Glu Glu Ile  
 225 230 235 240  
 40 Val Thr Lys Thr Val Arg Tyr Arg Thr Val Gly Asp Met Ser Cys Thr  
 245 250 255  
 Gly Ala Val Leu Ser Glu Ala Arg Thr Ile Asp Asp Val Ile Glu Glu  
 260 265 270  
 Ile Ala Thr Ser Thr Leu Thr Glu Arg Gly Ala Thr Arg Ala Asp Asp  
 275 280 285  
 45 Arg Leu Ser Glu Ser Ala Met Glu Asp Arg Lys Lys Glu Gly Tyr Phe  
 290 295 300

50 <210> 3  
 <211> 433  
 <212> PRT  
 <213> Corynebacterium glutamicum

55 <400> 3  
 Met Thr Ala Pro Thr Leu Asn Lys Ala Ser Glu Lys Ile Ala Ser Arg  
 1 5 10 15  
 Glu Thr Leu Arg Leu Cys Thr Ala Gly Ser Val Asp Asp Gly Lys Ser  
 20 25 30

Thr Phe Val Gly Arg Leu Leu His Asp Thr Lys Ser Val Leu Ala Asp  
 35 40 45  
 Gln Leu Ala Ser Val Glu Arg Thr Ser Ala Asp Arg Gly Phe Glu Gly  
 50 55 60  
 5 Leu Asp Leu Ser Leu Leu Val Asp Gly Leu Arg Ala Glu Arg Glu Gln  
 65 70 75 80  
 Gly Ile Thr Ile Asp Val Ala Tyr Arg Tyr Phe Ala Thr Asp Lys Arg  
 85 90 95  
 10 Thr Phe Ile Leu Ala Asp Thr Pro Gly His Val Gln Tyr Thr Arg Asn  
 100 105 110  
 Thr Val Thr Gly Val Ser Thr Ser Gln Val Val Val Leu Leu Val Asp  
 115 120 125  
 Ala Arg His Gly Val Val Glu Gln Thr Arg Arg His Leu Ser Val Ser  
 130 135 140  
 15 Ala Leu Leu Gly Val Arg Thr Val Ile Leu Ala Val Asn Lys Ile Asp  
 145 150 155 160  
 Leu Val Asp Tyr Ser Glu Glu Val Phe Arg Asn Ile Glu Lys Glu Phe  
 165 170 175  
 20 Val Gly Leu Ala Ser Ala Leu Asp Val Thr Asp Thr His Val Val Pro  
 180 185 190  
 Ile Ser Ala Leu Lys Gly Asp Asn Val Ala Glu Pro Ser Thr His Met  
 195 200 205  
 Asp Trp Tyr Thr Gly Pro Thr Val Leu Glu Ile Leu Glu Asn Val Glu  
 210 215 220  
 25 Val Ser His Gly Arg Ala His Asp Leu Gly Phe Arg Phe Pro Ile Gln  
 225 230 235 240  
 Tyr Val Ile Arg Glu His Ala Thr Asp Tyr Arg Gly Tyr Ala Gly Thr  
 245 250 255  
 30 Ile Asn Ala Gly Ser Val Ser Val Gly Asp Thr Val Tyr Leu Pro Glu  
 260 265 270  
 Gly Arg Thr Thr Gln Val Thr His Ile Asp Ser Ala Asp Gly Ser Leu  
 275 280 285  
 Gln Thr Ala Ser Val Gly Glu Ala Val Val Leu Arg Leu Ala Gln Glu  
 290 295 300  
 35 Ile Asp Leu Ile Arg Gly Glu Leu Ile Ala Gly Glu Asp Arg Pro Glu  
 305 310 315 320  
 Ser Val Arg Ser Phe Asn Ala Thr Val Val Gly Leu Ala Asp Arg Thr  
 325 330 335  
 40 Ile Lys Pro Gly Ala Ala Val Lys Val Arg Tyr Gly Thr Glu Leu Val  
 340 345 350  
 Arg Gly Arg Val Ala Ala Ile Glu Arg Val Leu Asp Ile Asp Gly Val  
 355 360 365  
 Asn Asp Asn Glu Ala Pro Glu Thr Tyr Gly Leu Asn Asp Ile Ala His  
 370 375 380  
 45 Val Arg Ile Asp Val Ala Gly Glu Leu Glu Val Glu Asp Tyr Ala Ala  
 385 390 395 400  
 Arg Gly Ala Ile Gly Ser Phe Leu Leu Ile Asp Gln Ser Ser Gly Asp  
 405 410 415  
 50 Thr Leu Ala Ala Gly Leu Val Gly His Arg Leu Arg Asn Asn Trp Ser  
 420 425 430  
 Ile

55 <210> 4  
 <211> 2170  
 <212> DNA  
 <213> Corynebacterium glutamicum

|    |  |     |
|----|--|-----|
|    | <220>  |     |
|    | <221> CDS  |     |
|    | <222> (271)..(1203)  |     |
| 5  | <223> cysK gene  |     |
|    | <220>  |     |
|    | <221> CDS  |     |
|    | <222> (1392)..(1955)   |     |
| 10 | <223> cysE gene  |     |
|    | <400> 4  |     |
|    | tccgacaacg gacttcttta aaagatgctt ttcgacgccg ctccccaacc attaaccccg  | 60  |
|    | cgagaaatat tcatcgaata gatgtcgatc tacctgcaaa tacgctcggt ctacaaataa  | 120 |
| 15 | tgaacagAAC tgtctacttt tcaaactgct ttttgtgtag actcaagtca cagaggccac  | 180 |
|    | ttcaagtaga tgtttcgtaa ttgtttacag cgtttacgca agcggtcgac caacaaaaaac | 240 |
| 20 | agcacttcaa tgattggagc accacccgac atg ggc aat gtg tac aac aac atc   | 294 |
|    | Met Gly Asn Val Tyr Asn Asn Ile                                    |     |
|    | 1 5  |     |
| 25 | acc gaa acc atc ggc cac acc cca ctg gta aag ctg aac aag ctc acc    | 342 |
|    | Thr Glu Thr Ile Gly His Thr Pro Leu Val Lys Leu Asn Lys Leu Thr    |     |
|    | 10 15 20   |     |
|    | gaa ggc ctc gac gca act gtc ctg gtc aag ctt gag tca ttc aac cca    | 390 |
| 30 | Glu Gly Leu Asp Ala Thr Val Leu Val Lys Leu Glu Ser Phe Asn Pro    |     |
|    | 25 30 35 40  |     |
|    | gca aac tcc gtc aag gac cgt atc ggt ctg gcc atc gtt gaa gat gca    | 438 |
|    | Ala Asn Ser Val Lys Asp Arg Ile Gly Leu Ala Ile Val Glu Asp Ala    |     |
|    | 45 50 55   |     |
| 35 | gag aag tcc ggt gca ctg aag cca ggc ggc acc atc gtt gaa gca acc    | 486 |
|    | Glu Lys Ser Gly Ala Leu Lys Pro Gly Gly Thr Ile Val Glu Ala Thr    |     |
|    | 60 65 70   |     |
| 40 | tcc ggc aac acc ggt atc gca ctg gca atg gtc ggc gct gca cgc gga    | 534 |
|    | Ser Gly Asn Thr Gly Ile Ala Leu Ala Met Val Gly Ala Ala Arg Gly    |     |
|    | 75 80 85   |     |
| 45 | tac aac gtt gtt ctc acc atg ccg gag acc atg tcc aac gag cgt cgc    | 582 |
|    | Tyr Asn Val Val Leu Thr Met Pro Glu Thr Met Ser Asn Glu Arg Arg    |     |
|    | 90 95 100  |     |
|    | gtt ctc ctc cgc gct tac ggt gca gag atc gtt ctt acc cca ggt gca    | 630 |
| 50 | Val Leu Leu Arg Ala Tyr Gly Ala Glu Ile Val Leu Thr Pro Gly Ala    |     |
|    | 105 110 115 120  |     |
|    | gca ggc atg cag ggt gca aag gac aag gca gac gaa atc gtc gct gaa    | 678 |
|    | Ala Gly Met Gln Gly Ala Lys Asp Lys Ala Asp Glu Ile Val Ala Glu    |     |
|    | 125 130 135  |     |
| 55 | cgc gaa aac gca gtc ctt gct cgc cag ttc gag aac gag gca aac cca    | 726 |
|    | Arg Glu Asn Ala Val Leu Ala Arg Gln Phe Glu Asn Glu Ala Asn Pro    |     |
|    | 140 145 150  |     |

|    |  |      |
|----|--|------|
|    | cgc gtc cac cgc gac acc acc gcg aag gaa atc ctc gaa gac acc gac      | 774  |
|    | Arg Val His Arg Asp Thr Thr Ala Lys Glu Ile Leu Glu Asp Thr Asp      |      |
|    | 155 160 165  |      |
| 5  | ggc aac gtt gat atc ttc gtt gca agc ttc ggc acc ggc gga acc gtc      | 822  |
|    | Gly Asn Val Asp Ile Phe Val Ala Ser Phe Gly Thr Gly Gly Thr Val      |      |
|    | 170 175 180  |      |
| 10 | acc ggc gtt ggc cag gtc ctg aag gaa aac aac gca gac gta cag gtc      | 870  |
|    | Thr Gly Val Gly Gln Val Leu Lys Glu Asn Asn Ala Asp Val Gln Val      |      |
|    | 185 190 195 200  |      |
| 15 | tac acc gtc gag cca gaa gcg tcc cca ctt ctg acc gct ggc aag gct      | 918  |
|    | Tyr Thr Val Glu Pro Glu Ala Ser Pro Leu Leu Thr Ala Gly Lys Ala      |      |
|    | 205 210 215  |      |
| 20 | ggt cca cac aag atc cag ggc atc ggc gca aac ttc atc ccc gag gtc      | 966  |
|    | Gly Pro His Lys Ile Gln Gly Ile Gly Ala Asn Phe Ile Pro Glu Val      |      |
|    | 220 225 230  |      |
|    | ctg gac cgc aag gtt ctc gac gac gtg ctg acc gtc tcc aac gaa gac      | 1014 |
|    | Leu Asp Arg Lys Val Leu Asp Asp Val Leu Thr Val Ser Asn Glu Asp      |      |
|    | 235 240 245  |      |
| 25 | gca atc gca ttc tcc cgc aag ctc gct acc gaa gag ggc atc ctc ggc      | 1062 |
|    | Ala Ile Ala Phe Ser Arg Lys Leu Ala Thr Glu Glu Gly Ile Leu Gly      |      |
|    | 250 255 260  |      |
| 30 | ggt atc tcc acc ggc gca aac atc aag gca gct ctt gac ctt gca gca      | 1110 |
|    | Gly Ile Ser Thr Gly Ala Asn Ile Lys Ala Ala Leu Asp Leu Ala Ala      |      |
|    | 265 270 275 280  |      |
| 35 | aag cca gag aac gct ggc aaa acc atc gtc acc gtt gtc acc gac ttc      | 1158 |
|    | Lys Pro Glu Asn Ala Gly Lys Thr Ile Val Thr Val Val Thr Asp Phe      |      |
|    | 285 290 295  |      |
| 40 | ggc gag cgc tac gtc tcc acc gtt ctt tac gaa gac atc cgc gac          | 1203 |
|    | Gly Glu Arg Tyr Val Ser Thr Val Leu Tyr Glu Asp Ile Arg Asp          |      |
|    | 300 305 310  |      |
| 45 | taattcttag cgactgttaa ccaactcaagc tctttgcttg ggtgggttttt tcatgtctca  | 1263 |
|    | aggtcggggtc ggggtgcgatt cgggtcgggtt ttgagtgtct ttgagtcctt ttaagtcctt | 1323 |
|    | ctttgcccggt gaataattct ctggatagtt tccacgtgca gttaagtcac gctgttagac   | 1383 |
| 50 | ttgcctgc atg ctc tcg aca ata aaa atg atc cgt gaa gat ctc gca aac     | 1433 |
|    | Met Leu Ser Thr Ile Lys Met Ile Arg Glu Asp Leu Ala Asn              |      |
|    | 315 320 325  |      |
|    | gct cgt gaa cac gat cca gca gcc cga ggc gat tta gaa aac gca gtg      | 1481 |
|    | Ala Arg Glu His Asp Pro Ala Ala Arg Gly Asp Leu Glu Asn Ala Val      |      |
|    | 330 335 340  |      |
| 55 | gtt tac tcc gga ctc cac gcc atc tgg gca cat cga gtt gcc aac agc      | 1529 |
|    | Val Tyr Ser Gly Leu His Ala Ile Trp Ala His Arg Val Ala Asn Ser      |      |
|    | 345 350 355  |      |



|    |   |      |
|----|---|------|
|    | tgg tgg aaa tcc ggt ttc cgc ggc ccc gcc cgc gta tta gcc caa ttc   | 1577 |
|    | Trp Trp Lys Ser Gly Phe Arg Gly Pro Ala Arg Val Leu Ala Gln Phe   |      |
|    | 360 365 370   |      |
| 5  | acc cga ttc ctc acc ggc att gaa att cac ccc ggt gcc acc att ggt   | 1625 |
|    | Thr Arg Phe Leu Thr Gly Ile Glu Ile His Pro Gly Ala Thr Ile Gly   |      |
|    | 375 380 385   |      |
| 10 | cgt cgc ttt ttt att gac cac gga atg gga atc gtc atc ggc gaa acc   | 1673 |
|    | Arg Arg Phe Phe Ile Asp His Gly Met Gly Ile Val Ile Gly Glu Thr   |      |
|    | 390 395 400 405   |      |
| 15 | gct gaa atc ggc gaa ggc gtc atg ctc tac cac ggc gtc acc ctc ggc   | 1721 |
|    | Ala Glu Ile Gly Glu Gly Val Met Leu Tyr His Gly Val Thr Leu Gly   |      |
|    | 410 415 420   |      |
| 20 | gga cag gtt ctc acc caa acc aag cgc cac ccc acg ctc tgc gac aac   | 1769 |
|    | Gly Gln Val Leu Thr Gln Thr Lys Arg His Pro Thr Leu Cys Asp Asn   |      |
|    | 425 430 435   |      |
|    | gtg aca gtc ggc gcg ggc gca aaa atc tta ggt ccc atc acc atc ggc   | 1817 |
|    | Val Thr Val Gly Ala Gly Ala Lys Ile Leu Gly Pro Ile Thr Ile Gly   |      |
|    | 440 445 450   |      |
| 25 | gaa ggc tcc gca att ggc gcc aat gca gtt gtc acc aaa gac gtg ccg   | 1865 |
|    | Glu Gly Ser Ala Ile Gly Ala Asn Ala Val Val Thr Lys Asp Val Pro   |      |
|    | 455 460 465   |      |
| 30 | gca gaa cac atc gca gtc gga att cct gcg gta gca cgc cca cgt ggc   | 1913 |
|    | Ala Glu His Ile Ala Val Gly Ile Pro Ala Val Ala Arg Pro Arg Gly   |      |
|    | 470 475 480 485   |      |
| 35 | aag aca gag aag atc aag ctc gtc gat ccg gac tat tac att           | 1955 |
|    | Lys Thr Glu Lys Ile Lys Leu Val Asp Pro Asp Tyr Tyr Ile           |      |
|    | 490 495   |      |
| 40 | taagaacagt tagcgcccta cctgaagttc aggcagggcg cttttttggg aagctccaga | 2015 |
|    | gtgcgtttgt tagccacgca ctagggacct ttaaccgtct aaaaccgccc ctgtgcgctt | 2075 |
|    | ctcagcacta cccgtgagaa ccacccccct gtgccagcta gttctttaga tccttatact | 2135 |
|    | cagggttctt ctgaatgaag ccagcgactg cagag                            | 2170 |
| 45 | <210> 5   |      |
|    | <211> 311   |      |
|    | <212> PRT   |      |
|    | <213> Corynebacterium glutamicum                                  |      |
| 50 | <400> 5   |      |
|    | Met Gly Asn Val Tyr Asn Asn Ile Thr Glu Thr Ile Gly His Thr Pro   |      |
|    | 1 5 10 15   |      |
| 55 | Leu Val Lys Leu Asn Lys Leu Thr Glu Gly Leu Asp Ala Thr Val Leu   |      |
|    | 20 25 30  |      |
|    | Val Lys Leu Glu Ser Phe Asn Pro Ala Asn Ser Val Lys Asp Arg Ile   |      |
|    | 35 40 45  |      |
|    | Gly Leu Ala Ile Val Glu Asp Ala Glu Lys Ser Gly Ala Leu Lys Pro   |      |
|    | 50 55 60  |      |



Gly Gly Thr Ile Val Glu Ala Thr Ser Gly Asn Thr Gly Ile Ala Leu  
 65 70 75 80  
 Ala Met Val Gly Ala Ala Arg Gly Tyr Asn Val Val Leu Thr Met Pro  
 85 90 95  
 5 Glu Thr Met Ser Asn Glu Arg Arg Val Leu Leu Arg Ala Tyr Gly Ala  
 100 105 110  
 Glu Ile Val Leu Thr Pro Gly Ala Ala Gly Met Gln Gly Ala Lys Asp  
 115 120 125  
 10 Lys Ala Asp Glu Ile Val Ala Glu Arg Glu Asn Ala Val Leu Ala Arg  
 130 135 140  
 Gln Phe Glu Asn Glu Ala Asn Pro Arg Val His Arg Asp Thr Thr Ala  
 145 150 155 160  
 Lys Glu Ile Leu Glu Asp Thr Asp Gly Asn Val Asp Ile Phe Val Ala  
 165 170 175  
 15 Ser Phe Gly Thr Gly Gly Thr Val Thr Gly Val Gly Gln Val Leu Lys  
 180 185 190  
 Glu Asn Asn Ala Asp Val Gln Val Tyr Thr Val Glu Pro Glu Ala Ser  
 195 200 205  
 20 Pro Leu Leu Thr Ala Gly Lys Ala Gly Pro His Lys Ile Gln Gly Ile  
 210 215 220  
 Gly Ala Asn Phe Ile Pro Glu Val Leu Asp Arg Lys Val Leu Asp Asp  
 225 230 235 240  
 Val Leu Thr Val Ser Asn Glu Asp Ala Ile Ala Phe Ser Arg Lys Leu  
 245 250 255  
 25 Ala Thr Glu Glu Gly Ile Leu Gly Gly Ile Ser Thr Gly Ala Asn Ile  
 260 265 270  
 Lys Ala Ala Leu Asp Leu Ala Ala Lys Pro Glu Asn Ala Gly Lys Thr  
 275 280 285  
 30 Ile Val Thr Val Val Thr Asp Phe Gly Glu Arg Tyr Val Ser Thr Val  
 290 295 300  
 Leu Tyr Glu Asp Ile Arg Asp  
 305 310  
  
 35 <210> 6  
 <211> 188  
 <212> PRT  
 <213> Corynebacterium glutamicum  
  
 40 <400> 6  
 Met Leu Ser Thr Ile Lys Met Ile Arg Glu Asp Leu Ala Asn Ala Arg  
 1 5 10 15  
 Glu His Asp Pro Ala Ala Arg Gly Asp Leu Glu Asn Ala Val Val Tyr  
 20 25 30  
 45 Ser Gly Leu His Ala Ile Trp Ala His Arg Val Ala Asn Ser Trp Trp  
 35 40 45  
 Lys Ser Gly Phe Arg Gly Pro Ala Arg Val Leu Ala Gln Phe Thr Arg  
 50 55 60  
 Phe Leu Thr Gly Ile Glu Ile His Pro Gly Ala Thr Ile Gly Arg Arg  
 65 70 75 80  
 Phe Phe Ile Asp His Gly Met Gly Ile Val Ile Gly Glu Thr Ala Glu  
 85 90 95  
 Ile Gly Glu Gly Val Met Leu Tyr His Gly Val Thr Leu Gly Gly Gln  
 100 105 110  
 55 Val Leu Thr Gln Thr Lys Arg His Pro Thr Leu Cys Asp Asn Val Thr  
 115 120 125  
 Val Gly Ala Gly Ala Lys Ile Leu Gly Pro Ile Thr Ile Gly Glu Gly  
 130 135 140

Ser Ala Ile Gly Ala Asn Ala Val Val Thr Lys Asp Val Pro Ala Glu  
 145 150 155 160  
 His Ile Ala Val Gly Ile Pro Ala Val Ala Arg Pro Arg Gly Lys Thr  
 165 170 175  
 5 Glu Lys Ile Lys Leu Val Asp Pro Asp Tyr Tyr Ile  
 180 185

10 <210> 7  
 <211> 1240  
 <212> DNA  
 <213> Corynebacterium glutamicum

15 <220>  
 <221> CDS  
 <222> (250)..(1032)  
 <223> cysH gene

20 <400> 7  
 tgggtgtgagt cttcggagga aacccaatcc caaccgcaac caccctctgt actgcccata 60  
 ctgcgcggga gaagttcttt tccccgatga gcaaacagaa ttcgcgtggg tgtgtgcgga 120  
 25 ttgcaccaga gtttttgaag tgaaatatca cggccaggac gatccagtgc acaggccagc 180  
 accagcaaag tccacatcgc aagcattaaa agaattctctc gaaagacaca aaagaggtga 240  
 gtcgcaaca atg agc ttt caa cta gtt aac gcc ctg aaa aat act ggt tcg 291  
 30 Met Ser Phe Gln Leu Val Asn Ala Leu Lys Asn Thr Gly Ser  
 1 5 10  
 gta aaa gat ccc gag atc tca ccc gaa gga cct cgc acg acc aca ccg 339  
 35 Val Lys Asp Pro Glu Ile Ser Pro Glu Gly Pro Arg Thr Thr Thr Pro  
 15 20 25 30  
 ttg tca cca gag gta gca aaa cat aac gag gaa ctc gtc gaa aag cat 387  
 Leu Ser Pro Glu Val Ala Lys His Asn Glu Glu Leu Val Glu Lys His  
 35 40 45  
 40 gct gct gcg ttg tat gac gcc agc gcg caa gag atc ctg gaa tgg aca 435  
 Ala Ala Ala Leu Tyr Asp Ala Ser Ala Gln Glu Ile Leu Glu Trp Thr  
 50 55 60  
 45 gcc gag cac gcg ccg ggc gct att gca gtg acc ttg agc atg gaa aac 483  
 Ala Glu His Ala Pro Gly Ala Ile Ala Val Thr Leu Ser Met Glu Asn  
 65 70 75  
 acc gtg ctg gcg gag ctg gct gcg cgg cac ctg ccg gaa gct gat ttc 531  
 50 Thr Val Leu Ala Glu Leu Ala Ala Arg His Leu Pro Glu Ala Asp Phe  
 80 85 90  
 ctc ttt ttg gac acc ggt tac cac ttc aag gag acc ctt gaa gtt gcc 579  
 55 Leu Phe Leu Asp Thr Gly Tyr His Phe Lys Glu Thr Leu Glu Val Ala  
 95 100 105 110  
 cgt cag gta gat gag cgc tat tcc cag aag ctt gtc acc gcg ctg ccg 627  
 Arg Gln Val Asp Glu Arg Tyr Ser Gln Lys Leu Val Thr Ala Leu Pro  
 115 120 125

|    |   |      |
|----|---|------|
|    | atc ctc aag cgc acg gag cag gat tcc att tat ggt ctc aac ctg tac   | 675  |
|    | Ile Leu Lys Arg Thr Glu Gln Asp Ser Ile Tyr Gly Leu Asn Leu Tyr   |      |
|    | 130 135 140   |      |
| 5  | cgc agc aac cca gcg gcg tgc tgc cga atg cgc aaa gtt gaa ccg ctg   | 723  |
|    | Arg Ser Asn Pro Ala Ala Cys Cys Arg Met Arg Lys Val Glu Pro Leu   |      |
|    | 145 150 155   |      |
| 10 | gcg gcg tcg tta agc cca tac gct ggc tgg atc acc ggc ctg cgc cgc   | 771  |
|    | Ala Ala Ser Leu Ser Pro Tyr Ala Gly Trp Ile Thr Gly Leu Arg Arg   |      |
|    | 160 165 170   |      |
| 15 | gct gat ggc cca acc cgt gct caa gcc cct gcg ctg agc ttg gat gcc   | 819  |
|    | Ala Asp Gly Pro Thr Arg Ala Gln Ala Pro Ala Leu Ser Leu Asp Ala   |      |
|    | 175 180 185 190   |      |
| 20 | acc ggc agg ctc aag att tct cca att atc acc tgg tca ttg gag gaa   | 867  |
|    | Thr Gly Arg Leu Lys Ile Ser Pro Ile Ile Thr Trp Ser Leu Glu Glu   |      |
|    | 195 200 205   |      |
| 25 | acc aac gag ttc att gcg gac aac aac ctc atc gat cac cca ctt acc   | 915  |
|    | Thr Asn Glu Phe Ile Ala Asp Asn Asn Leu Ile Asp His Pro Leu Thr   |      |
|    | 210 215 220   |      |
| 30 | cat cag ggt tat cca tca att gga tgc gaa acc tgc acc ctt cct gtt   | 963  |
|    | His Gln Gly Tyr Pro Ser Ile Gly Cys Glu Thr Cys Thr Leu Pro Val   |      |
|    | 225 230 235   |      |
| 35 | gct gaa gga caa gac cct agg gcc ggc cgt tgg gct gga aac gcc aag   | 1011 |
|    | Ala Glu Gly Gln Asp Pro Arg Ala Gly Arg Trp Ala Gly Asn Ala Lys   |      |
|    | 240 245 250   |      |
| 40 | aca gaa tgc gga ctt cac tca tgaccacaac cgttgcatca gtactatccc      | 1062 |
|    | Thr Glu Cys Gly Leu His Ser                                       |      |
|    | 255 260   |      |
|    | cacaccttaa agatcttgaa aatgaatcca tccacatcct ccgcgaggta gctggccagt | 1122 |
| 45 | ttgataaggt cggcctgctg ttttccggcg gtaaggattc cgtcgtggtg tacgagcttg | 1182 |
|    | cgcgccgcgc tttcgctcca gctaacgtgc cttttgaatt gctgcacgtg gacaccgg   | 1240 |
| 50 | <210> 8   |      |
|    | <211> 261   |      |
|    | <212> PRT   |      |
|    | <213> Corynebacterium glutamicum                                  |      |
| 55 | <400> 8   |      |
|    | Met Ser Phe Gln Leu Val Asn Ala Leu Lys Asn Thr Gly Ser Val Lys   |      |
|    | 1 5 10 15   |      |
|    | Asp Pro Glu Ile Ser Pro Glu Gly Pro Arg Thr Thr Thr Pro Leu Ser   |      |
|    | 20 25 30  |      |
|    | Pro Glu Val Ala Lys His Asn Glu Glu Leu Val Glu Lys His Ala Ala   |      |
|    | 35 40 45  |      |

Ala Leu Tyr Asp Ala Ser Ala Gln Glu Ile Leu Glu Trp Thr Ala Glu  
           50                          55                          60  
 5 His Ala Pro Gly Ala Ile Ala Val Thr Leu Ser Met Glu Asn Thr Val  
    65                          70                          75                          80  
 Leu Ala Glu Leu Ala Ala Arg His Leu Pro Glu Ala Asp Phe Leu Phe  
                           85                          90                          95  
 10 Leu Asp Thr Gly Tyr His Phe Lys Glu Thr Leu Glu Val Ala Arg Gln  
                           100                          105                          110  
 Val Asp Glu Arg Tyr Ser Gln Lys Leu Val Thr Ala Leu Pro Ile Leu  
                           115                          120                          125  
 15 Lys Arg Thr Glu Gln Asp Ser Ile Tyr Gly Leu Asn Leu Tyr Arg Ser  
                           130                          135                          140  
 20 Asn Pro Ala Ala Cys Cys Arg Met Arg Lys Val Glu Pro Leu Ala Ala  
    145                          150                          155                          160  
 Ser Leu Ser Pro Tyr Ala Gly Trp Ile Thr Gly Leu Arg Arg Ala Asp  
                           165                          170                          175  
 25 Gly Pro Thr Arg Ala Gln Ala Pro Ala Leu Ser Leu Asp Ala Thr Gly  
                           180                          185                          190  
 Arg Leu Lys Ile Ser Pro Ile Ile Thr Trp Ser Leu Glu Glu Thr Asn  
                           195                          200                          205  
 30 Glu Phe Ile Ala Asp Asn Asn Leu Ile Asp His Pro Leu Thr His Gln  
                           210                          215                          220  
 35 Gly Tyr Pro Ser Ile Gly Cys Glu Thr Cys Thr Leu Pro Val Ala Glu  
    225                          230                          235                          240  
 Gly Gln Asp Pro Arg Ala Gly Arg Trp Ala Gly Asn Ala Lys Thr Glu  
                           245                          250                          255  
 40 Cys Gly Leu His Ser  
                           260

45 <210> 9  
     <211> 28  
     <212> DNA  
     <213> Artificial sequence

50 <220>  
     <223> Description of the artificial sequence: Primer  
           cysDex1

55 <400> 9  
     ctggtaccgc ggacttcact catgacca

<210> 10  
 <211> 28

<212> DNA  
<213> Artificial sequence

5 <220>  
<223> Description of the artificial sequence: Primer  
cysDex2

10 <400> 10  
cgtctagagg aacctgcggt gcacagac 28

15 <210> 11  
<211> 28  
<212> DNA  
<213> Artificial sequence

20 <220>  
<223> Description of the artificial sequence: Primer  
cysEex1

<400> 11  
ctggtacctc acgctgtag acttgcct 28

25 <210> 12  
<211> 28  
<212> DNA  
<213> Artificial sequence

30 <220>  
<223> Description of the artificial sequence: Primer  
cysEex2

35 <400> 12  
gatctagaac aaacgcactc tggagctt 28

40 <210> 13  
<211> 28  
<212> DNA  
<213> Artificial sequence

45 <220>  
<223> Description of the artificial sequence: Primer  
cysHex1

50 <400> 13  
acggtacctg agtcgcaaca atgagctt 28

55 <210> 14  
<211> 28  
<212> DNA  
<213> Artificial sequence

<220>  
<223> Description of the artificial sequence: Primer  
cysHex2

<400> 14  
gttctagacg gaggatgtgg atggattc 28

5 <210> 15  
<211> 28  
<212> DNA  
<213> Artificial sequence

10 <220>  
<223> Description of the artificial sequence: Primer  
cysKex1

15 <400> 15  
agggtaccca agcggtcgac caacaaaa 28

20 <210> 16  
<211> 28  
<212> DNA  
<213> Artificial sequence

25 <220>  
<223> Description of the artificial sequence: Primer  
cysKex2

30 <400> 16  
cttctagaat tagtcgcgga tgtcttcg 28

[ ]

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number  
**WO 02/29029 A3**

(51) International Patent Classification<sup>7</sup>: **C12N 9/12**,  
9/88, 9/10, 9/02, 1/21, C12P 13/04, 13/12, C12Q 1/68,  
A23K 1/16

(21) International Application Number: **PCT/EP01/09723**

(22) International Filing Date: 23 August 2001 (23.08.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
100 48 603.7 30 September 2000 (30.09.2000) DE  
101 09 691.7 28 February 2001 (28.02.2001) DE  
101 36 986.7 28 July 2001 (28.07.2001) DE

(71) Applicant: **DEGUSSA AG** [DE/DE]; Bennigsenplatz 1,  
40474 Düsseldorf (DE).

(72) Inventors: **FARWICK, Mike**; Gustav-Adolf-Strasse  
11, 33615 Bielefeld (DE). **HUTHMACHER, Klaus**;  
Lärchenweg 18, 63584 Gelnhausen (DE). **SCHISCHKA,**  
**Natalie**; Stuttgarter Strasse 1, 33659 Bielefeld (DE).  
**BATHE, Brigitte**; Twieten 1, 33154 Salzkotten (DE).  
**PFEFFERLE, Walter**; Jahnstrasse 33, 33790 Halle  
(Westf.) (DE). **BINDER, Michael**; Kalberkamp 28,  
33803 Steinhagen (Westf.) (DE). **GREISSINGER, Di-**  
**eter**; Augasse 1f, 61194 Niddatal (DE). **THIERBACH,**  
**Georg**; Gunststrasse 21, 33613 Bielefeld (DE).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI,  
SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA,  
ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian  
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European  
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,  
IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF,  
CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD,  
TG).

**Published:**

- with international search report
- before the expiration of the time limit for amending the  
claims and to be republished in the event of receipt of  
amendments

(88) Date of publication of the international search report:  
13 June 2002

*For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.*

(54) Title: **NUCLEOTIDE SEQUENCES WHICH CODE FOR THE CYSD, CYSN, CYSK, CYSE AND CYSH GENES OF C.  
GLUTAMICUM**

(57) Abstract: The invention provides nucleotide sequences from coryneform bacteria which code for the *cysD*, *cysN*, *cysK*, *cysE*  
and *cysH* genes and a process for the fermentative preparation of amino acids using bacteria in which the genes mentioned are  
enhanced, a process for the fermentative preparation of L-amino acids using coryneform bacteria in which at least the *cysD* gene, the  
*cysN* gene, the *cysK* gene, the *cysE* gene and/or the *cysH* gene is present in enhanced form, and the use of polynucleotides which  
contain the sequences according to the invention as hybridization probes and a process for the preparation of an L-methionine-  
containing animal feedstuffs additive from fermentation broths.

WO 02/29029 A3





# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/09723

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N9/12 C12N9/88 C12N9/10 C12N9/02 C12N1/21  
C12P13/04 C12P13/12 C12Q1/68 A23K1/16

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, SEQUENCE SEARCH, BIOSIS

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| X          | WO 97 15673 A (CONSORTIUM ELEKTROCHEM IND<br>;LEINFELDER WOLFRED (DE); HEINRICH PETE)<br>1 May 1997 (1997-05-01)                         | 28, 43, 44            |
| Y          | page 2, paragraph 2  | 1-45                  |
| Y          | EP 1 006 192 A (DEGUSSA)<br>7 June 2000 (2000-06-07)<br>paragraph '0017!   | 1-45                  |
| X          | DATABASE EBI 'Online!<br>EMBL;<br>MMCV_STRLA, 30 May 2000 (2000-05-30)<br>XP002191266<br>70% identity with Seq ID 2 in 293 aa<br>overlap | 1-7                   |



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

### \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- \*G\* document member of the same patent family

Date of the actual completion of the international search

25 February 2002

Date of mailing of the international search report

03/04/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2,  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Stolz, B

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/09723

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| P, X       | <p>WO 01 00842 A (BASF AG)<br/> 4 January 2001 (2001-01-04)<br/> -&amp; DATABASE EBI 'Online!<br/> AAH68058,<br/> XP002191267<br/> 100% identity with Seq ID 1 in 912 nt<br/> overlap (232-1142:1-912)<br/> -&amp; DATABASE EBI 'Online!<br/> AAF71139,<br/> XP002191268<br/> 100% identity with Seq ID 1 in 980 nt<br/> overlap (1488-2467:1-980)<br/> -&amp; DATABASE EBI 'Online!<br/> AAF71310,<br/> XP002191269<br/> 100% identity with Seq ID 7 in 541 nt<br/> overlap (150-690:1-541)<br/> ---</p>  | 1-45                  |
| P, X       | <p>WO 01 00843 A (BASF AG)<br/> 4 January 2001 (2001-01-04)<br/> -&amp; DATABASE EBI 'Online!<br/> AX064763,<br/> XP002191270<br/> 100% identity with Seq ID 7 in 541 nt<br/> overlap (150-690:1-541)<br/> -&amp; DATABASE EBI 'Online!<br/> AX063961,<br/> XP002191271<br/> 100% identity with seq ID 4 in 669 nt<br/> overlap (1310-1978:1-669)<br/> -&amp; DATABASE EBI 'Online!<br/> AX063963,<br/> XP002191272<br/> 99.8% identity with Seq ID 4 in 1056 nt<br/> overlap (171-1226:1-1056)<br/> ---</p> <p style="text-align: center;">-/--</p> | 1-45                  |

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 01/09723

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
|------------|--|-----------------------|
| P,X        | <p>EP 1 108 790 A (KYOWA HAKKO KOGYO KK)<br/> 20 June 2001 (2001-06-20)<br/> -&amp; DATABASE EBI 'Online!<br/> AAH68057,<br/> XP002191273<br/> 100% identity with seq ID 1 in 1299 nt<br/> overlap (1146-2444:1-1299)<br/> -&amp; DATABASE EBI 'Online!<br/> AAH68058,<br/> XP002191274<br/> 100% identity with Seq ID 7 in 209 nt<br/> overlap (1032-1240:1-209)<br/> -&amp; DATABASE EBI 'Online!<br/> AAH68059,<br/> XP002191275<br/> 100% identity with Seq ID 7 in 693 nt<br/> overlap (340-1032:1-693)<br/> -&amp; DATABASE EBI 'Online!<br/> AAH67783,<br/> XP002191276<br/> 100% identity with Seq ID in 546 nt<br/> overlap (1410-1955:1-546)<br/> -&amp; DATABASE EBI 'Online!<br/> AAH67782,<br/> XP002191277<br/> 100% identity with seq ID 4 in 924 nt<br/> overlap (289-1203:1-924)<br/> -----</p> | 1-45                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 01/09723

| Patent document<br>cited in search report |   | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---|---------------------|----------------------------|---------------------|
| WO 9715673                                | A | 01-05-1997          | DE 19539952 A1             | 30-04-1997          |
|   |   |                     | AT 211175 T                | 15-01-2002          |
|   |   |                     | BR 9610910 A               | 13-07-1999          |
|   |   |                     | CA 2235752 A1              | 01-05-1997          |
|   |   |                     | CN 1200764 A               | 02-12-1998          |
|   |   |                     | CZ 9801269 A3              | 15-07-1998          |
|   |   |                     | DE 59608521 D1             | 31-01-2002          |
|   |   |                     | WO 9715673 A1              | 01-05-1997          |
|   |   |                     | EP 0858510 A1              | 19-08-1998          |
|   |   |                     | HU 9900078 A2              | 28-04-1999          |
|   |   |                     | JP 2000504926 T            | 25-04-2000          |
|   |   |                     | PL 327187 A1               | 23-11-1998          |
|   |   |                     | US 6218168 B1              | 17-04-2001          |
| EP 1006192                                | A | 07-06-2000          | DE 19855313 A1             | 08-06-2000          |
|   |   |                     | BR 9905776 A               | 24-04-2001          |
|   |   |                     | CN 1256314 A               | 14-06-2000          |
|   |   |                     | EP 1006192 A2              | 07-06-2000          |
|   |   |                     | HU 9904447 A2              | 28-11-2000          |
|   |   |                     | JP 2000228990 A            | 22-08-2000          |
|   |   |                     | SK 163399 A3               | 11-07-2000          |
|   |   |                     | US 6184007 B1              | 06-02-2001          |
|   |   |                     | ZA 9907406 A               | 08-06-2000          |
| WO 0100842                                | A | 04-01-2001          | AU 5420500 A               | 31-01-2001          |
|   |   |                     | WO 0100842 A2              | 04-01-2001          |
|   |   |                     | WO 0100843 A2              | 04-01-2001          |
|   |   |                     | AU 5421600 A               | 31-01-2001          |
|   |   |                     | WO 0100805 A2              | 04-01-2001          |
|   |   |                     | AU 5559000 A               | 31-01-2001          |
|   |   |                     | WO 0100844 A2              | 04-01-2001          |
|   |   |                     | AU 5421300 A               | 31-01-2001          |
|   |   |                     | AU 5836900 A               | 31-01-2001          |
|   |   |                     | WO 0100804 A2              | 04-01-2001          |
| WO 0100843                                | A | 04-01-2001          | AU 5421300 A               | 31-01-2001          |
|   |   |                     | WO 0100843 A2              | 04-01-2001          |
|   |   |                     | AU 5559000 A               | 31-01-2001          |
|   |   |                     | WO 0100844 A2              | 04-01-2001          |
|   |   |                     | AU 5836900 A               | 31-01-2001          |
|   |   |                     | WO 0100804 A2              | 04-01-2001          |
|   |   |                     | AU 5421600 A               | 31-01-2001          |
|   |   |                     | WO 0100805 A2              | 04-01-2001          |
|   |   |                     | AU 5420500 A               | 31-01-2001          |
|   |   |                     | WO 0100842 A2              | 04-01-2001          |
| EP 1108790                                | A | 20-06-2001          | EP 1108790 A2              | 20-06-2001          |